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Aniline Acetate
Aniline Hydrochloride
*Benzil
*Benzophenone
Camphoric Acid
Caprylic Alcohol
Dextrose, Pure
Dimethyl Aminoazobenzol
Dimethyl Aniline
Dimethyl Glyoxime
Ethyl Iodide
Ethyl Oxalate
*Glycocoll
Hexachlorbenzol
*Hydroxylamine Hydrochloride
*Hydroxylamine Sulphate
Malonic Ester
Methyl Ethyl Ketone
Methyl Iodide
Methyl Oxalate
Methyl Orange
Methyline Iodide
Monobrom Naphthaline
Nitroso Dimethylaniline
Nitroso B Naphthol
Phenacetolin
Phenylacetic Acid
*Phenylacetate
Phenyl Hydrazine
Phenyl Hydrazine Hydrochloride
*Quinoline
Salicylic Aldehyde
Sodium Benzol Sulphonate
Sodium Phenylate
*Urea Nitrate
Uric Acid

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CEPHALIN.

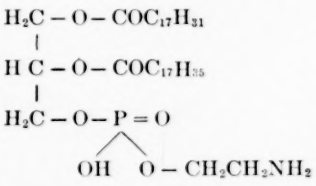
VII. THE GLYCEROPHOSPHORIC ACID OF CEPHALIN.

By P. A. LEVENE and IDA P. ROLF.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, August 2, 1919.)

The generally accepted structural formula of cephalin is as follows:



This conception presumes the existence of glycerophosphoric acid in the molecule of cephalin. Thudichum,[1] who discovered cephalin, also was the first to make this assumption. Every other worker who followed Thudichum made similar assumptions. Parnas[2] was the first to call attention to the lack of evidence in favor of the theory, though admitting the possibility of its correctness. MacLean in his monograph on "Lecithin and allied substances" presents a very lucid summary of the weak points of the theory.

The main objection to the conclusions of the older writers lies in the fact that the material employed by them was not a pure cephalin but a mixture of this substance with lecithin.

In recent years, Fränkel and Dimitz[3] claimed to have identified glycerophosphoric acid as a component of cephalin. Unfortunately the evidence presented by these investigators is not con-

[1] Thudichum, J. L. W., The chemical constitution of the brain, London, 1884.

[2] Parnas, J., *Biochem. Z.*, 1909, xxii, 411.

[3] Fränkel, S., and Dimitz, L., *Biochem. Z.*, 1909, xxi, 337.

vincing since they have failed to substantiate their claim with satisfactory analytical data. The analytical data are limited to one element; namely, barium.

Regardless of the meager proof of the nature of their substance, Fränkel and Dimitz have laid claim to a second very important discovery; namely, that the glycerophosphoric acid derived from cephalin was the optical isomer of the corresponding substance occurring in lecithin.

Willstätter and Lüdecke[4] found that the glycerophosphoric acid obtained from lecithin was optically active and levorotary. The substance obtained by Fränkel and Dimitz was dextrorotary, and hence followed the claim that it was isomeric to the substance described by Willstätter and Lüdecke.

The presence of glycerophosphoric acid in the cephalin molecule seemed *a priori* quite probable in the light of facts discovered in recent years. Simultaneously, Foster[5] and Levene and West[6] have shown that glycerol is present in cephalin in the quantity required by the generally accepted theory.

Furthermore, it was found that the basic part of cephalin is amino ethyl alcohol, and that in this base the alcoholic group only is substituted, the amino group remaining free.

Theoretically there are only two alternative ways in which glycerol may be attached to the phosphoric acid, either directly (forming glycerophosphoric acid) or indirectly. If the linking were indirect, then the glycerol would be attached to the aminoethanol. If that were so, then the amino group could not be free in the molecule of cephalin. Thus the presence of glycerophosphoric acid in cephalin could have been accepted even before the substance was actually isolated, although for the final proof, the isolation of the substance would have been desirable. On the other hand, without isolating the substance there was no way to reach a decision as to which of the possible isomers of glycerophosphoric acid is the one present in the molecule of cephalin. True, the optical rotation of hydrolecithin and of hydrocephalin is practically identical in direction and magnitude. Levene and West[7] have pointed out that this fact may be

[4] Willstätter, R., and Lüdecke, K., *Ber. chem. Ges.*, 1904, xxxvii, 3753.
[5] Foster, M. L., *J. Biol. Chem.*, 1915, xx, 403.
[6] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 50.
[7] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxv, 287.

interpreted to signify that the glycerophosphoric acids in the two substances are identical. This conclusion contradicts the theory of Fränkel and Dimitz. Hence it became necessary to choose between the two views, and, in order to make the choice intelligently, an effort was made to isolate the glycerophosphoric acid of the cephalin molecule.

Glycerophosphoric Acid from Lecithin.

The present communication contains a report on the preparation of glycerophosphoric acid from both cephalin and lecithin. The latter was prepared for the following reasons. First, Willstätter and Lüdecke[4] stated that the magnitude of the optical rotation of the glycerophosphoric acid from lecithin differed depending on the mode of preparation. Second, the directions for the preparation of the substances given by these authors are so meager that any one repeating the work could have no assurance that he was following exactly the conditions of Willstätter and Lüdecke. Under such circumstances if the glycerophosphoric acid prepared by us from cephalin was found different from the one of Willstätter and Lüdecke, the difference could have been ascribed not to the inherent properties of the substance, but to the manner of its preparation.

The glycerophosphoric acid obtained by us from lecithin resembled the one obtained by Willstätter and Lüdecke, in respect to the direction of its rotation. It was levorotary. However, the magnitude of the rotation of our substance was lower than the maximum rotation found by the earlier workers. The differences are probably due to differences in handling, since all the samples were partially racemized.

The maximum rotation found by us was $[\alpha]_D^{20} = -0.74$, by the other writers $[\alpha]_D^{20} = -1.71$.

In a general way the crude substance was prepared in the manner indicated by Willstätter and Lüdecke. However, for purification it was found convenient to convert the barium salt into the lead salt, to purify the lead salt, and then to reconvert this into the barium salt.

As a rule, the hydrolysis was brought about at room temperature; however, for the sake of comparison one experiment was

performed by boiling the hydrolysis mixture. This variation in the condition of hydrolysis remained without influence on the rotatory power of the resulting substance.

Hydrolysis of Cephalin.

After the most favorable conditions of hydrolysis of lecithin had been established, they were applied to the hydrolysis of cephalin. The result was practically identical. The glycerophosphoric acid obtained from cephalin, purified through conversion of the crude barium salt into the lead salt with subsequent reconversion of the latter into the barium salt, possessed the optical rotation of the same magnitude as the acid from lecithin; namely, $[\alpha]_D^{20} = -0.69$.

In this respect the result obtained by us is new. It establishes the fact that glycerophosphoric acid enters into the structure of cephalin, and further that the acid is identical with that present in lecithin. However, it is worthy of note that while the crude barium glycerophosphate obtained from lecithin always showed a levorotation, though of a comparatively low magnitude, the barium glycerophosphate from cephalin showed dextrorotation. The magnitude of this rotation was progressively descending on purification of the substance. All the dextrorotary samples were found to contain nitrogen, and the purification of the substance which led to the fall of the dextrorotation also led to the diminution in the nitrogen content. It is probable, therefore, that the claim of Fränkel and Dimitz to the discovery of a dextrorotary glycerophosphoric acid from cephalin was an error brought about by the fact that the substance analyzed by them was a mixture of glycerophosphoric acid with some product of intermediary hydrolysis.

EXPERIMENTAL.

Barium Glycerophosphoric Acid from Lecithin.

Hydrolysis at Low Temperature.

75 gm. of lecithin were obtained by the decomposition with ammonium carbonate of lecithin cadmium chloride containing 20 per cent amino nitrogen. This material was ground under water until a uniform emulsion was obtained, and then shaken

for 6 hours at room temperature with 1 liter of a saturated aqueous solution of barium hydroxide (the equivalent of $2\frac{1}{4}$ mols of $Ba(OH)_2$). After standing for 3 hours, the mixture was filtered and the excess of barium contained in the filtrate removed quantitatively with sulfuric acid. The resulting filtrate was concentrated to a small bulk under diminished pressure, and the barium salt precipitated by pouring the thin syrup into several volumes of absolute alcohol. After one purification by dissolving in a small quantity of water, filtering through bone-black, and reprecipitating with alcohol, the yield of this material was 7.5 gm.

The barium salt obtained in this way was subjected to two more purifications by solution in water and precipitation by alcohol. Finally it was twice dissolved in a minimum amount of water and allowed to stand until it separated as a mass of heavy granules and non-crystalline scales. These were filtered with suction, washed with alcohol, and dried under diminished pressure. Obtained in this manner, the salt, when dry, is a light tan, granular material, which dissolves in water giving a clear yellow solution. Though very soluble in cold water it is not hygroscopic; on warming the cold solution a fine amorphous powder is precipitated which does not dissolve completely as the solution is cooled. It is insoluble in alcohol, ether, and acetone. All rotations were made in 20 per cent aqueous solution. A sample (No. 23) had the following composition. It contained no nitrogen.

0.1018 gm. of substance dried under diminished pressure at the temperature of zylene vapor gave on combustion 0.0472 gm. of CO_2, 0.0248 gm. of H_2O, and 0.0712 gm. of ash.

0.2000 gm. of substance containing 7.36 per cent moisture gave 0.0666 mg. of $Mg_2P_2O_7$.

0.1000 gm. of substance containing 7.36 per cent moisture gave 0.0668 mg. of $BaSO_4$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 23.
	per cent	*per cent*
C	11.71	12.64
H	2.29	2.72
P	10.10	10.02
Ba	44.68	42.43

The rotation of the substance was as follows:

$$[\alpha]_D^{20} = \frac{-0.075 \times 100}{1 \times 18.42} = -0.41.$$

As our investigation progressed, we found that the method of reprecipitation described above was not effective for the complete purification of the salts, as shown by their continued retention of nitrogen, and the process described below was applied to all our later products.

2 gm. of substance (No. 23) were dissolved in water. A 25 per cent aqueous solution of neutral lead acetate was added until precipitation was complete; the precipitate was filtered and thoroughly washed with water, alcohol, and ether. The lead salt thus isolated was suspended in water, and hydrogen sulfide passed through until the decomposition was complete. The lead sulfide was removed by filtration and the filtrate concentrated under diminished pressure to a small volume and made slightly alkaline to litmus with barium hydroxide. From the concentrated solution the barium salt was precipitated by adding several volumes of 95 per cent alcohol. The yield of this preparation, No. 63, was 1.5 gm.

When rapidly precipitated by alcohol, the barium salt separates in white amorphous flakes, which dry to a gleaming white powder. The color reported in the foregoing experiment is apparently due to an impurity as all samples obtained analytically pure were white when dry, and very readily soluble in water, giving colorless, water-clear solutions. If the salt after filtration is not dried either by washing with alcohol and ether or by rapid desiccation, the amorphous flakes undergo an apparent fusion and dry to a mass of colorless granules and scales. This granular form can also be obtained by precipitating the salt from a very concentrated aqueous solution by a gradual addition of alcohol.

0.101 gm. of substance dried under diminished pressure at temperature of xylene vapor gave on combustion 0.0438 gm. of CO_2, 0.0194 gm. of H_2O, and 0.0742 gm. of ash.

0.2786 gm. of substance containing 9.11 per cent water gave 0.0912 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 63.
	per cent	*per cent*
C	11.71	11.84
H	2.29	2.15
P	10.10	10.00
Ba	44.68	44.03

The rotation of No. 63 is

$$[\alpha]_D^{20} = \frac{-0.11 \times 100}{1 \times 18.82} = -0.58.$$

Both granular and flocculent forms after air-drying contain water of crystallization, the value of which is not constant, though in general it approaches $1\frac{3}{4}$ mols of H_2O. The nature of the substance, however, does not permit one to attribute too much importance to this value. After preliminary drying under diminished pressure over sulfuric acid at room temperature, the loss of water on complete drying was more constant, corresponding to $C_3H_7O_6PBa \cdot H_2O$.

After air-drying, on desiccation over sulfuric acid under diminished pressure at the temperature of xylene vapor, 0.1108 gm. of No. 63 lost 0.0098 gm. of H_2O.

	Calculated for $C_3H_7O_6PBa \cdot 1\frac{1}{4}H_2O$.	Found. No. 63.
	per cent	*per cent*
H_2O	9.30	8.84

The substance was dried for 24 to 48 hours under diminished pressure over sulfuric acid at room temperature. On further drying to constant weight at the temperature of xylene vapor, 0.1100 gm. of No. 56 lost 0.0060 gm. of H_2O and 0.1092 gm. of No. 57 lost 0.0058 gm. of H_2O.

	Calculated for $C_3H_7O_6PBa \cdot H_2O$.	Found.	
		No. 56.	No. 57.
	per cent	*per cent*	*per cent*
H_2O	5.53	5.45	5.31

In order to be certain of the source of the glycerophosphoric acid, lecithin cadmium chloride was isolated from egg oil by the method recommended by Levene and West[8] for the preparation of pure lecithin. The amino content of this material was negligible. From its cadmium salt the lecithin was isolated by treatment with $(NH_4)_2CO_3$ in boiling 85 per cent alcoholic solution.

[8] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxiv, 175.

75 gm. of this product were hydrolyzed with 1 liter of barium hydroxide solution, and the barium salt was isolated in the same manner as No. 23. 8 gm. of crude material were obtained from which the material most insoluble in alcohol was separated by repeated solution in water and fractional precipitation with small volumes of alcohol. After eighteen such reprecipitations, a substance (No. 55) of the following composition and analysis was obtained. It contained no nitrogen.

0.200 gm. of substance containing 5.03 per cent moisture gave 0.0706 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 5.03 per cent moisture gave 0.0690 gm. of $BaSO_4$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 55
	per cent	*per cent*
N	0.00	0.00
P	10.10	10.36
Ba	44.68	42.75

$$[\alpha]_D^{20} = \frac{-0.12 \times 100}{1 \times 18.89} = -0.63.$$

That a longer period of hydrolysis would give a better yield and not affect the rotation adversely seemed probable, and was proved by hydrolyzing 100 gm. of lecithin for 16 hours with 1,350 cc. of a saturated barium hydroxide solution. When isolated by the method described for No. 23 the yield of the crude material was 35 gm. (90 per cent of the theory). After three precipitations from alcohol this material (No. 56) had the following composition.

0.200 gm. of substance containing 5.45 per cent moisture gave 0.0572 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 5.45 per cent moisture gave 0.0664 gm. of $BaSO_4$.

0.200 gm. of substance containing 5.45 per cent moisture used for Kjeldahl nitrogen determination required 0.26 cc. of 0.1 N HCl.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 56.
	per cent	*per cent*
N	0.00	0.18
P	10.10	8.43
Ba	44.68	41.33

No. 56 was then further purified by precipitating the lead salt and decomposing this by the directions given above. The

resulting substance (No. 57) had the following composition and rotation. It contained no nitrogen.

0.200 gm. of substance containing 5.31 per cent moisture gave 0.0704 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 5.31 per cent moisture gave 0.0680 gm. of $BaSO_4$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 57.
	per cent	*per cent*
N	0.00	0.00
P	10.10	10.38
Ba	44.68	42.26

$$[\alpha]_D^{20} = \frac{-0.09 \times 100}{1 \times 19.36} = -0.46.$$

A second purification through the lead salt resulted in No. 60, whose rotation was

$$[\alpha]_D^{20} = \frac{-0.14 \times 100}{1 \times 18.92} = -0.74.$$

A third purification by this method gave no further change in the rotation.

The final analysis after an additional precipitation with alcohol follows.

0.1026 gm. of substance dried *in vacuo* at temperature of xylene vapor gave on combustion 0.0482 gm. of CO_2, 0.0248 gm. of H_2O, and 0.0774 gm. of ash.

0.200 gm. of substance containing 7.06 per cent moisture gave 0.0696 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 78.
	per cent	*per cent*
C	11.71	12.81
H	2.29	2.70
P	10.10	10.43
Ba	44.68	44.87

Hydrolysis at Higher Temperature.

97 gm. of lecithin cadmium chloride were dissolved in 250 cc. of boiling 50 per cent alcohol and this solution was added to one containing 103 gm. ($3\frac{1}{4}$ mols) of barium hydroxide in 1 liter of hot water. After 1 hour of gentle boiling the mixture was cooled and filtered. The excess of barium hydroxide was removed

from the filtrate by quantitative precipitation with sulfuric acid, and the filtered solution concentrated in vacuum to small bulk. The addition of an equal volume of 95 per cent alcohol precipitated the salt of glycerophosphoric acid, but the barium chloride formed in the course of the hydrolysis remained in solution. Thorough washing with 50 per cent alcohol removed all traces of chlorides from the filtered barium glycerophosphoric acid. After two preliminary precipitations by alcohol, the salt was converted into the lead salt, decomposed, and again reprecipitated as the barium salt by the directions given above.

The specific rotation of this substance was

$$[\alpha]_D^{20} = \frac{-0.03 \times 100}{1 \times 18.89} = -0.16.$$

A second purification by conversion into the lead salt raised this rotation as follows:

$$[\alpha]_D^{20} = \frac{-0.12 \times 100}{1 \times 18.89} = -0.63.$$

On analysis 0.102 gm. (No. 69), dried under diminished pressure over sulfuric acid at temperature of xylene vapor, gave on combustion 0.0234 gm. of H_2O, 0.0449 gm. of CO_2, and 0.0724 gm. of ash.

0.300 gm. (No. 69), containing 7.77 per cent moisture, gave 0.1021 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 69.
	per cent	*per cent*
C	11.71	12.00
H	2.29	2.56
P	10.10	10.28
Ba	44.68	41.19

Barium Glycerophosphoric Acid from Cephalin.

Preparation of Cephalin.

Three samples of cephalin were used as the source of the glycerophosphoric salts described below. All were obtained from ox brains by six extractions with ether containing 5 per cent water. This material, after evaporation of the ether, was precipitated with acetone and the fats and saturated phosphatides were removed from the precipitate by repeatedly dissolving in ether,

removing all material insoluble in ether at 0°, and reprecipitating by pouring into acetone. From the mixture of lecithin and cephalin thus obtained, the cephalin was separated by precipitating the ethereal solution with alcohol. The first sample of cephalin was purified by repeated precipitation by alcohol from ethereal solution and finally three precipitations from a solution in gasoline (B. P. 50–60°). The analysis of this material (No. 44) is given below.

The second sample, after three precipitations from an ethereal solution by alcohol, was shaken with water until a uniform emulsion was obtained. From this the cephalin was precipitated by dilute hydrochloric acid, separated by centrifuging, and thoroughly washed with acetone. Further purification was effected by repeatedly dissolving either in ether (saturated with water at room temperature) or in gasoline (B. P. 50–60°) and precipitating with alcohol. This material (No. 58) had the composition indicated below.

The third sample was prepared in the following manner. The crude cephalin was exhaustively extracted with alcohol at room temperature and then repeatedly reprecipitated by alcohol from ether or gasoline solution, until its amino content was that indicated in the analysis (No. 135) recorded below. Following this the material was emulsified with water, precipitated by hydrochloric acid, and the precipitate washed with alcohol.

No. 44.

0.5 gm. was dissolved in 5 cc. of glacial acetic acid.

2 cc. of this solution for Kjeldahl determination required 340 cc. of 0.1 N HCl = 0.00468 gm. of N.

2 cc. of this solution for Van Slyke determination gave 8.55 cc. of N at $T° = 26°$C. and $P = 757.1$ mm.; N = 0.00476 gm.

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{1}{1}$$

No. 58.

2 gm. of cephalin were hydrolyzed with HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution required for Kjeldahl determination 0.90 cc. of 0.1 N HCl = 0.00126 gm. of N.

2 cc. of this solution by Van Slyke determination gave 0.91 cc. of N at $T° = 24°$ and $P = 755.9$ mm.; N = 0.000505 gm.

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{1}{1}$$

No. 135.

0.2 gm. was dissolved in 10 cc. of glacial acetic acid.

5 cc. of this solution for Kjeldahl determination required 1.27 cc. of 0.1 N HCl = 0.001778 gm. of N.

1 cc. of this solution for Van Slyke determination gave 0.65 cc. of N at $T° = 30°$ and $P = 759.2$ mm.; N = 0.003505 gm.

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{1}{1}$$

Hydrolysis of Cephalin.

75 gm. of cephalin (No. 44) were hydrolyzed by shaking for 9 hours at room temperature with a liter of saturated barium hydroxide solution. The directions given for No. 23 were followed in working up the resulting mixture. On pouring the concentrated aqueous solution into alcohol a colloidal solution formed which was precipitated by the addition of a few cc. of an aqueous solution of barium acetate. After thirteen reprecipitations by alcohol from its aqueous solution, this material (No. 51) had the following composition and rotation.

0.200 gm. of substance containing 5.67 per cent moisture gave 0.0556 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 5.67 per cent moisture gave 0.0662 gm. of $BaSO_4$.

0.200 gm. of substance used for Kjeldahl determination required 1.12 cc. of 0.1 N HCl.

	Calculated for $C_3H_7O_8PBa$.	Found. No. 51.
	per cent	*per cent*
N	0.00	0.83
P	10.10	8.21
Ba	44.68	41.28

$$[\alpha]_D^{20} = \frac{+0.15 \times 100}{1 \times 18.88} = +0.80.$$

No. 51 five times reprecipitated by alcohol still retained nitrogen.

0.200 gm. of substance (No. 53) containing 9.28 per cent moisture required for Kjeldahl determination 0.77 cc. of 0.1 N HCl.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 53.
	per cent	*per cent*
N	0.00	0.59

Its rotation was

$$[\alpha]_D^{20} = \frac{+0.08 \times 100}{1 \times 18.20} = +0.44.$$

Ten additional reprecipitations by alcohol gave a substance (No. 59) of the following composition and rotation.

0.200 gm. of substance containing 9.07 per cent moisture gave 0.0566 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 9.07 per cent moisture gave 0.0644 gm. of $BaSO_4$.

0.200 gm. of substance containing 9.07 per cent moisture used for Kjeldahl determination required 0.57 cc. of 0.1 N HCl.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 59.
	per cent	*per cent*
N	0.00	0.40
P	10.10	8.67
Ba	44.68	41.68

$$[\alpha]_D^{20} = \frac{+0.04 \times 100}{1 \times 18.19} = +0.22.$$

All these salts agreed in property and appearance with the salts derived from the hydrolysis of lecithin by the same sort of purification. They all retained a distinctly yellow color which could not be wholly removed by bone-blacking the aqueous solution. All specimens were very soluble in water and rotations were made on 20 per cent aqueous solutions.

The mother liquors from the purifications of No. 51 and its derivatives were concentrated in vacuum to a small bulk and precipitated with alcohol. The bulky precipitate was filtered and again dissolved in water. To this was added a 25 per cent aqueous solution of neutral lead acetate, and on standing lead glycerophosphoric acid settled out. This salt was filtered, suspended in water, and decomposed by hydrogen sulfide. The precipitated lead sulfide was filtered and the filtrate concentrated to a small bulk. The barium salt of glycerophosphoric acid was again isolated by adding barium hydroxide until slightly alkaline to litmus and precipitating with several volumes of alcohol. This

material was a glistening white, amorphous powder, identical in all characteristics with No. 63 obtained in an analogous manner from lecithin. Its composition and rotation were as follows. It contained no nitrogen.

0.1594 gm. of substance containing 5.56 per cent moisture gave 0.0548 gm. of $Mg_2P_2O_7$.

0.103 gm. of substance containing 5.56 per cent moisture gave 0.0752 gm. of $BaSO_4$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 64.
	per cent	*per cent*
P	10.10	10.14
Ba	44.68	45.48

$$[\alpha]_D^{20} = \frac{-0.13 \times 100}{1 \times 18.97} = -0.69.$$

Another hydrolysis of cephalin was made on 75 gm. of cephalin (No. 58) which were shaken for 16 hours at room temperature with $2\frac{1}{4}$ mols of barium hydroxide. The barium glycerophosphoric acid was isolated by the method used in the previous experiments, and after a preliminary precipitation with alcohol, was purified by conversion to the lead salt, and reprecipitation as the barium salt, according to the directions given under the foregoing experiment. After two additional precipitations by alcohol from its aqueous solution, the yield of this material (No. 68) was 5 gm. and its composition and rotation were the following.

0.101 gm. of substance dried under diminished pressure over sulfuric acid at temperature of xylene vapor yielded on combustion 0.0520 gm. of CO_2, 0.0218 gm. of H_2O, and 0.0684 gm. of ash.

0.200 gm. of substance containing 7.84 per cent moisture gave 0.0626 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 68.
	per cent	*per cent*
C	11.77	14.04
H	2.29	2.41
P	10.10	9.92
Ba	44.68	39.12

$$[\alpha]_D^{20} = \frac{-0.9 \times 100}{1 \times 18.40} = -0.49.$$

A second purification through the lead salt and one reprecipitation from alcohol gave a substance (No. 77) of the following composition and rotation.

0.099 gm. of substance dried under diminished pressure over sulfuric acid in xylene bath gave on combustion 0.0484 gm. of Mg_2CO_2, 0.0222 gm. of H_2O, and 0.0692 gm. of ash.

0.300 gm. of substance containing 9.00 per cent moisture gave 0.0956 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 77.
	per cent	*per cent*
C	11.71	13.33
H	2.29	2.50
P	10.10	9.76
Ba	44.68	41.36

$$[\alpha]_D^{20} = \frac{-0.12 \times 100}{1 \times 18.00} = -0.67.$$

A third hydrolysis of cephalin was carried out in the same way on 50 gm. of cephalin (No. 135) and the purification of this material was exactly like that giving rise to No. 77. The analysis and rotation of this material were as follows:

0.1011 gm. of substance dried under diminished pressure over sulfuric acid in xylene bath, gave on combustion 0.044 gm. of CO_2, 0.0216 gm. of H_2O, and 0.0724 gm. of ash.

0.200 gm. of substance containing 8.80 per cent moisture gave 0.0648 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 145.
	per cent	*per cent*
C	11.71	11.86
H	2.29	2.40
P	10.10	9.89
Ba	44.68	43.88

$$[\alpha]_D^{20} = \frac{-0.13 \times 100}{1 \times 20.05} = -0.65$$

Like the barium glycerophosphates obtained from lecithin, these salts, after air-drying, contained variable amounts of water of crystallization approaching $1\frac{3}{4}$ mols; while after desiccation under diminished pressure for about 24 hours at room temperature, a more constant content of water was retained, corresponding to $C_3H_7O_6PBa \cdot H_2O$.

On complete desiccation over H_2SO_4 under diminished pressure at the temperature of zylene vapor, the following results were obtained.

0.1256 gm. of No. 59, previously air-dried, lost 0.0114 gm. of H_2O.
0.1088 " " " 77, " " " 0.0098 " " H_2O.
0.1104 " " " 78, " " " 0.0093 " " H_2O.

	Calculated for $C_3H_7O_6PBa \cdot 1\frac{1}{2}H_2O$.	Found. No. 59.	No. 77.	No. 78.
	per cent	*per cent*	*per cent*	*per cent*
H_2O	9.30	9.07	9.00	9.29

The substance dried at room temperature under diminished pressure lost on drying to constant weight at the temperature of xylene vapor as follows:

0.1164 gm. of No. 51 lost 0.0066 gm. of H_2O.
0.1116 " " " 64 " 0.0062 " " H_2O.

	Calculated for $C_3H_7O_6PBa \cdot H_2O$.	Found. No. 51.	No. 64.
	per cent	*per cent*	*per cent*
H_2O	5.53	5.67	5.56

COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

III. FATE OF P-NITROPHENYLACETIC ACID IN THE ORGANISM OF FOWL, DOG, AND MAN.

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Many organic substances have been fed to animals to determine the process by which the animal body will dispose of them. If non-toxic, they may be wholly or at least in part oxidized and utilized for heat production. If toxic, such compounds must be detoxicated and eliminated as rapidly as possible. In the latter case we are able to study with a certain degree of precision what might be called the non-specific defense mechanism of the body.

We have already studied the method employed by the body when certain putrefactive compounds derived from phenylalanine and tyrosine are detoxicated and eliminated in the urine. Oxidation and reduction play a large part in this process of detoxication, but often a point is reached where β-oxidation of the side chain is no longer possible, and in this case conjugation seems to be the method most often employed by the animal body. Thus benzoic acid no longer subject to oxidation in the organism is coupled with glycocoll and eliminated in the urine as hippuric acid. Again, phenylacetic acid, which results from the action of bacteria on phenylalanine, cannot be oxidized by the tissues but is detoxicated by combining it with one of the shorter amino-acids. In this case the formation of the phenylacetic acid in the body of man and the lower animals seems to be the same type of reaction, but the process of detoxication is apparently more complicated in the animal than that taking place in the human body.

After feeding phenylacetic acid to animals such as dogs (1), rabbits (2), and monkeys (3), the acid appears in the urine com-

bined with glycocoll, as phenaceturic acid; when ingested by human beings (4), phenylacetic acid is excreted in the urine in combination with glutamine, as phenylacetylglutamine. It has also been shown (5) that there is a difference in the processes of detoxication employed by man and animals after the feeding of *p*-hydroxybenzoic acid and *p*-hydroxyphenylacetic acid.

In the literature, one finds little concerning the metabolism of nitro compounds and particularly aromatic nitro derivatives. Sieber and Smirnow (6) fed large doses of *p*-nitrobenzaldehyde to a dog and recovered small amounts of *p*-nitrohippuric acid urea from the dog's urine. Cohn (7) fed *p*-nitrobenzaldehyde to rabbits and isolated from their urine a compound made up of *p*-nitrobenzoic acid and *p*-acetylaminobenzoic acid. Jaffe (8) fed "large amounts" (30 gm.) of *p*-nitrotoluene to a dog. He recovered some of the substance unchanged from the feces, some from the urine as *p*-nitrobenzoic acid, but most of it he found was eliminated in the urine as *p*-nitrohippuric acid urea. Bertagnini (9) ingested small quantities of nitrobenzoic acid at frequent intervals, thus ingesting in all 80 gm. during a period of several days. He was unable to prove the presence of either nitrobenzoic or nitrohippuric acid in the urine, but extracted from the urine an oily substance which gave nitrobenzoic acid and glycocoll on hydrolysis.

As there seemed to be no definite information in the literature regarding the fate of either the nitrobenzoic acids or nitrophenylacetic acids in the human body we decided to undertake some feeding experiments which eventually included not only man but the dog and fowl also. The compound used in these experiments was in each case *p*-nitrophenylacetic acid.

As oxidation of the side chain of phenylacetic acid seems impossible for either the animal or human body, there were several changes which this compound might undergo when ingested by a person. It seemed most probable that the nitro group should remain untouched and that a combination with glutamine or glycocoll should be formed, and perhaps urea should also be added to the *p*-nitrophenylacetylglutamine or *p*-nitrophenaceturic acid thus formed. On the other hand, a reduction of the nitro group was not impossible with a secondary acetylation of the amino group.

In the case of the dog it seemed most probable that *p*-nitrophenaceturic acid or *p*-nitrophenaceturic acid urea would be formed and eliminated in the urine.

The fowl seems to furnish, in most cases, large quantities of ornithine for the detoxication of foreign substances instead of glycocoll. Thus after feeding benzoic acid to chickens Jaffe (10) isolated from the urine a substance composed of 1 mol of ornithine and 2 mols of benzoic acid which he called ornithuric acid. Totani (11) after feeding phenylacetic acid to hens recovered a substance from the urine which he termed phenacetornithuric acid, and which was analogous to the compound isolated by Jaffe as it consisted of 2 mols of phenylacetic acid and 1 mol of ornithine.

Fate of p-Nitrophenylacetic Acid in the Human Organism.

The subject was a student of 70 kilos body weight and apparently in the best of health. The acid, being insoluble in water, could not be taken in the form of solution nor in capsules on account of bulk. A dose of 5 gm. of acid in crystalline form was accurately weighed out, taken into the mouth, and washed down with copious quantities of water. The acid possesses a very disagreeable taste not unlike pepper, with an after taste not unlike that of the copper salts.

The urine was collected for 48 hours after each dose of the acid, each collection was carefully neutralized with sodium carbonate as soon as voided, then the entire amount slowly evaporated on a water bath at low temperature. The urine when evaporated to a thick syrup was cooled below room temperature and acidified with H_3PO_4 until it showed an acid reaction with Congo red. This concentrated urine was placed in a continuous extracting apparatus and extracted for 5 hour periods with ether. The different ether extracts were placed in the ice box for a number of days to allow the formation of crystals. As no crystals appeared, the ether extracts were evaporated somewhat each day, then placed on ice over night. During this time no crystals appeared, so all the ether extracts were united and allowed to evaporate slowly at room temperature. As dryness was reached, only a yellow oil appeared and no crystalline substance. This

oily residue was dissolved in hot water, boiled with charcoal, filtered, and then allowed to cool. After a short time long yellow needles appeared in the solution and later the solution took on the form of a crystalline mass. The crystals were removed by suction and recrystallized several times from hot water. Dried at 80° they melted at 150–151.5.° The melting point as well as the crystalline form showed the substance to be *p*-nitrophenylacetic acid which had been eliminated in the urine unchanged.

Analysis.—0.1533 gm. of the substance required 8.60 cc. of 0.1 N sulfuric acid (Kjeldahl).

	Calculated for $C_8 H_7 N O_4$. *per cent*	Found. *per cent*
N.....	7.74	7.84

Only *p*-nitrophenylacetic acid crystallized out of the water solution of the ether extract, so it was evaporated to a small volume and repeatedly extracted with ether to remove the last traces of *p*-nitrophenylacetic acid. Enough concentrated sulfuric acid was added to this water solution to produce a 30 per cent acid solution and the solution boiled under a reflux condenser for several hours. The acid solution was cooled and repeatedly extracted with ether to remove any *p*-nitrophenylacetic acid which might be present. The ether extracts were united, evaporated to dryness, and the residue dissolved in hot water. No trace of *p*-nitrophenylacetic acid appeared at any stage in this extract so it seemed that no compound of *p*-nitrophenylacetic acid had been extracted from the urine by the ether.

It seemed reasonable to expect a compound of *p*-nitrophenylacetic acid with either glycocoll or glutamine so the evaporated urine was again extracted repeatedly with alcohol. As *p*-nitrophenaceturic acid is quite insoluble in cold alcohol this substance should have crystallized out of the alcohol if present, but no crystals appeared. The alcoholic extracts were united and evaporated, but only large amounts of urea and some hippuric acid crystals appeared. The alcoholic extract was evaporated to dryness and dissolved in hot water, and the water solution acidified with sulfuric acid and boiled under a reflux condenser for several hours. This acid solution was cooled and extracted with ether but again no *p*-nitrophenylacetic acid was found. It appeared that no compound of *p*-nitrophenylacetic acid had been extracted

from the urine by the alcohol. The urine was further extracted with ethyl acetate and lastly with benzene following the same method as employed in the alcohol extraction, but no trace of the acid could be found. As a last resort a part of the evaporated urine was diluted with enough water to bring it into solution and strongly acidified with sulfuric acid. After boiling this for several hours it was cooled and extracted with ether to determine the amount of *p*-nitrophenylacetic acid which might have been split off from any compound existing in the urine. Again none of the acid appeared, so there certainly could have been no compound in the urine containing *p*-nitrophenylacetic acid.

After two 5 gm. doses of the acid had been ingested, a total of 6.87 gm. (68.70 per cent) of *p*-nitrophenylacetic acid was recovered from the urine as the uncombined acid.

It seems peculiar that this acid should be found free in the urine. While perhaps less toxic than phenylacetic acid, still some symptoms of intoxication such as nausea, headache, and diarrhea were experienced by the subject. It is certainly more irritating to the mucous membranes than benzoic acid, and benzoic acid taken in corresponding doses is almost quantitatively converted into hippuric acid.

Fate of p-Nitrophenylacetic Acid in the Organism of the Dog.

A dog of 32.70 kilos body weight was fed three doses of *p*-nitrophenylacetic acid. The acid crystals were pulverized and packed into large gelatin capsules. The capsules were inserted into large chunks of cooked meat which were thrown to the dog and swallowed without mastication. The first dose of the acid consisted of 5 gm.; a second dose of 5 gm. was fed 1 week later, followed by a third dose of 7 gm. 2 days later.

The dog was kept in a large metabolism cage and the urine collected for 48 hours after each dose of the acid. The different portions of urine collected during this time were united and evaporated to a thick syrup, acidified with H_2SO_4, and extracted with ether for several hours in a continuous extracting apparatus. All portions of the ether used in this extraction were united and the ether was distilled off. The residue was dissolved in cold absolute ether, filtered, and the filtrate allowed to evaporate at room temperature. As no crystals appeared until the point of dryness was reached, the residue was redissolved in hot water,

boiled with charcoal, and allowed to stand in the ice box for 24 hours. Long yellow needles appeared in the solution and finally became almost a solid mass. The crystals were removed by suction and recrystallized from hot water. Dried at 80–90°, the crystals melted at 150–151.5°. The melting point as well as the characteristic solubility and crystalline structure of the substance showed it to be *p*-nitrophenylacetic acid which had been excreted in the urine unchanged. After a dose of 5 gm., 1.53 gm. of the acid were recovered from the urine while, after a dose of 5 gm. followed after 48 hours by a dose of 7 gm., 5.32 gm. of the *p*-nitrophenylacetic acid were isolated from the dog's urine.

The evaporated urine was removed from the ether-extracting apparatus and placed in a large separatory funnel. It was then extracted several times with large volumes of hot alcohol. The alcoholic extracts were united and slowly evaporated to dryness. The residue was dissolved in hot water, boiled with charcoal, and filtered. The filtrate was slowly evaporated by stages until crystals appeared on cooling. This substance which seemed quite insoluble in water was thus easily separated from urea, hippuric acid, and other alcohol-soluble urinary constituents. Recrystallized from hot water, the substance formed extremely long hair-like needles. When dry, the substance melted at 172–173°. The melting point, as well as the solubility and crystalline form of the substance, showed it to be identical with the *p*-nitrophenaceturic acid prepared synthetically by Hotter (12).

0.2761 gm. of substance required 11.45 cc. of 0.1 N NaOH for neutralization.

Calculated for $C_{10}H_{10}N_2O_5$: required 11.60 cc. of 0.1 N NaOH.

2 gm. of the substance were boiled for 2 hours with 30 per cent HCl, cooled, and the acid solution was extracted several times with ether. The ether extracts were evaporated to dryness and the residue was dissolved in hot water and boiled with charcoal. After filtering, the filtrate was allowed to stand for some hours and crystals of *p*-nitrophenylacetic acid appeared. These crystals when dried melted at 150–151°. The amount of *p*-nitrophenaceturic acid isolated from the urine after a 5 gm. dose of *p*-nitrophenylacetic acid was 0.757 gm., while after a dose of 5 gm. followed by 7 gm. of the acid, 2.873 gm. of *p*-nitrophenaceturic acid were recovered.

The fate of *p*-nitrophenylacetic acid in the organism of the dog is similar to that of its homologue *p*-nitrobenzoic acid.

The *p*-nitrotoluene fed to dogs (8) was first converted into *p*-nitrobenzoic acid, followed by a secondary reaction in which most of this substance was converted into *p*-nitrohippuric acid urea. The greater part of the *p*-nitrophenylacetic acid was excreted in the urine uncombined while a part of it was conjugated with glycocoll and excreted as *p*-nitrophenaceturic acid. None of the latter compound, however, was found in combination with urea. After feeding 5 gm. of the *p*-nitrophenylacetic acid, 1.53 gm. (30.60 per cent) were excreted in the urine unchanged, while only 0.757 gm. (15.14 per cent) was excreted as the glycocoll compound. Thus a total of only 2.287 gm. (45.64 per cent) of the substance were recovered from the urine. After feeding 5 gm. followed by 7 gm. of the substance, 5.32 gm. (44.35 per cent) were isolated from the urine as the free acid and 2.873 gm. were obtained from the urine as the glycocoll compound (*p*-nitrophenaceturic acid). Therefore after a total of 12 gm. had been fed, 61.47 per cent of the substance was recovered.

Fate of p-Nitrophenylacetic Acid in the Organism of the Fowl.

For this work a hen of 2.18 kilos body weight was selected. The acid was administered by means of a funnel and soft rubber tube. In this case a solution of the sodium salt was used and washed down with large quantities of water. The dose in each case amounted to 1 gm. of the acid. The substance proved quite toxic, as the hen refused to eat for some days after the first dose and showed marked signs of depression. The hen was kept in a metabolism cage and all excreta saved for 48 hours after each dose. The excreta, which were always very hard and dry, were stirred into a paste after the addition of sufficient amounts of water. To this mass dilute sulfuric acid was added until the reaction was strongly acid to Congo red. It was then immediately extracted with ethyl acetate alcohol (10 : 1) mixture in a continuous extracting apparatus. The first and second extracts were evaporated to one-fifth the original volume *in vacuo* at 40°, then placed on ice for 48 hours. This process was repeated until the extracts became very concentrated but no crystals appeared. After complete evaporation, there remained a black tar-like mass. This substance seemed to be entirely insoluble in cold water but

slightly soluble in hot water. On cooling the hot water solution, it became a milky emulsion and under the microscope was found to contain fine droplets of an oil-like substance held in suspension. On cooling to room temperature the liquid became clear with a light yellow, oily deposit on the sides of the receptacle. There appeared to be two different substances present, one an oil-like substance and a second black gum-like substance which was unaffected by water. The water was poured off and both substances were dried at 40°. The mixture was extracted for 12 hours with ether in a continuous extracting apparatus. Most of the black gummy substance remained in the extracting apparatus but changed in color to a dull gray. The yellow substance dissolved easily in ether. The ether extract was filtered and allowed to evaporate at room temperature, leaving a yellow crystalline deposit. This crystalline mass was dissolved in hot water and slowly cooled. Long yellow crystals appeared in the water solution. The crystals were removed and dried at 80°. The melting point was between 149–150°, so the substance was apparently *p*-nitrophenylacetic acid.

0.2018 gm. of the substance required 11.10 cc. of 0.1 N sulfuric acid (Kjeldahl).

	Calculated for $C_8H_7NO_4$. *per cent*	Found. *per cent*
N	7.74	7.70

The residue from the ether extract was dissolved in warm alcohol and allowed to cool; as no crystals appeared, ether was added until the solution became turbid. The flask was tightly stoppered and placed in the ice box for 24 hours. A fine deposit of microscopic crystals was found on the bottom of the flask so these were filtered off and more ether was added. By repeating this process, enough of the material was gathered for further work. This substance appeared to be insoluble in water but easily soluble in alkaline solutions. The substance was dissolved in barium hydroxide and the excess of barium removed by passing CO_2 through the solution and filtering off the barium carbonate. The filtrate containing the barium salt of the substance was evaporated by stages to one-third its original volume. After standing in the ice box for several days, large leaf-like crystals appeared on the surface of the liquid and formed a solid crust. The potassium and sodium

salts of the acid were formed from the barium salt by adding a solution of sodium and potassium sulfate to the solution of the barium salt. These salts were found to be too hydroscopic for analysis. The water solutions of all the salts formed were found to be dextrorotatory.

To prepare the pure substance a solution of the barium salt was acidified with H_2SO_4 and extracted with alcohol. The alcohol solution was concentrated and a few drops of ether were added until the solution became turbid. After standing for several days in the ice box, short, thick, irregular needles appeared in the solution. These were recrystallized from an alcohol ether mixture and dried *in vacuo*. The dried substance melted at 184–185°.

The analysis gave the following results.

0.1321 gm. of the substance gave 0.2662 gm. of CO_2 and 0.0576 gm. of H_2O.

0.2371 gm. of the substance required 20.80 cc. of 0.1 N sulfuric acid (Kjeldahl).

Calculated for $C_{21}H_{22}N_4O_8$: C = 54.99 per cent, H = 4.84 per cent, N = 12.22 per cent.

Found. C = 54.95 per cent, H = 4.99 per cent, and N = 12.30 per cent.

0.1191 gm. of the substance required 2.70 cc. of 0.1 N sodium hydroxide for neutralization. Calculated for $C_{21}H_{22}N_4O_8$: 2.60 cc. of 0.1 N NaOH were required.

A weighed amount of the substance was boiled for 3 hours with a 30 per cent solution of HCl, cooled, and extracted with ether. The ether extracts were evaporated to dryness and the residue recrystallized from hot water. The resulting yellow crystals when dried melted at 148.5–150°.

0.1007 gm. of the substance required 5.45 cc. of 0.1 N sulfuric acid (Kjeldahl).

Calculated for $C_8H_7NO_4$: N = 7.74 per cent.

Found. N = 7.58 per cent.

This substance was *p*-nitrophenylacetic acid. The acid solution remaining after the ether extraction was evaporated *in vacuo* to drive off the excess of HCl. The residue was dissolved in a small amount of water and made alkaline with sodium carbonate. To this alkaline solution benzoyl chloride was added in small amounts at frequent intervals. During the entire period the solution was kept alkaline, shaken vigorously after each addition of benzoyl

chloride, and cooled as often as necessary. After 45 minutes the solution was removed from the separatory funnel and acidified with HCl. The acidified solution was extracted several times with ether to remove all benzoic acid formed. After standing for some time a mass of leaf-like crystals appeared. These crystals were washed chlorine-free and dissolved in warm alcohol. By cooling the solution leaf-like crystals of ornithuric acid appeared. The substance was pure ornithuric acid as was shown by the melting point of 184–185° and by the following analysis.

0.1262 gm. of the substance required 7.55 cc. of 0.1 N sulfuric acid (Kjeldahl).

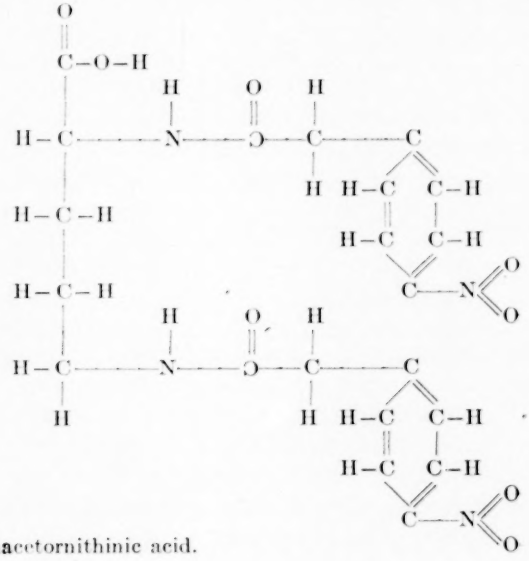
0.1136 gm. of the substance gave 0.2801 gm. of CO_2 and 0.0624 gm. of H_2O.

Calculated for $C_{19}H_{20}N_2O_4$: C = 67.02 per cent, H = 5.92 per cent, and N = 8.23 per cent.

Found. C = 67.23 per cent, H = 6.13 per cent, and N = 8.37 per cent.

This compound was identical with the ornithuric acid prepared by Jaffe (13), thus proving ornithine as the other constituent of the compound isolated from the excreta of the hen.

This compound must consist of 1 mol of ornithine and 2 mols of *p*-nitrophenylacetic acid and therefore has the following formula.



P-nitrophenacetornithinic acid.

The combination of *p*-nitrophenylacetic acid with ornithine is quite in line with the results obtained by other investigators in experiments where fowls were used. In this case as in the experiments performed on the human being and on the dog no reduction or alteration of the nitro group was found in any case, as was reported by Cohn (7) after feeding rabbits *p*-nitrobenzaldehyde.

CONCLUSION.

1. *p*-Nitrophenylacetic acid was ingested by a man in 5 gm. doses. 68.70 per cent of the acid was recovered from the urine in an uncombined state. No compound of the acid was found in the urine.

2. *p*-Nitrophenylacetic acid was fed to a dog in 5 and 7 gm. doses. 61.47 per cent of the acid was isolated from the urine. Of this amount 44.35 per cent was in the uncombined state while 17.12 per cent was combined with glycocoll and excreted as *p*-nitrophenaceturic acid.

3. *p*-Nitrophenylacetic acid was fed to a hen in 1 gm. doses. Some of the acid was excreted uncombined but the greater portion was conjugated with ornithine, and excreted as *p* nitrophenacetornithinic acid. This acid which had not been previously prepared was isolated, analyzed, and its structure determined. The barium, potassium, and sodium salts of the compound were prepared and found to be dextrorotatory.

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STUDIES OF BLOOD REGENERATION.*

I. EFFECT OF HEMORRHAGE ON ALKALINE RESERVE.

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INTRODUCTION.

With the exception of Milroy's[1] work, no systematic attempt has been made, as far as the writer has been able to ascertain, to follow chemically the changes in alkaline reserve during the period immediately following hemorrhage. Since the Van Slyke[2] method has been devised, such a study has been greatly facilitated. Not only can more extensive data be obtained by the use of this method, due to its simplicity, but also the results so obtained have greater significance due to the fact that changes can be detected too small to influence the hydrogen ion concentration which Milroy determined.

The hemorrhages which Milroy[1] studied with cats and dogs were large (approximately one-third the total volume of blood). In all cases he found a distinct rise in the hydrogen ion concentration of the plasma, determined by the gas chain method; *i.e.*, a loss of reserve alkali. This change was most apparent in the cases in which the plasma was subjected to high concentrations of CO_2. Samples of blood drawn at intervals of 15 and 45 minutes after hemorrhage showed these distinct changes in reaction. Milroy concluded that there must have been rapid compensatory passage of the tissue fluids into the circulation, the fluid which first entered the circulation being extremely poor in reserve alkali. The greatest increase in hydrogen ion concentration was found after the 15 minute interval.

* The work described in this article forms part of a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Wisconsin.

[1] Milroy, T. H., *J. Physiol.*, 1917, li, 259.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Since hemorrhage uncomplicated by other conditions such as shock and anesthesia is rarely encountered clinically many studies of these conditions are closely interwoven. Cannon and his coworkers[3] have studied clinically many cases of primary and secondary wound shock, hemorrhage, etc., with and without other complications, in a casualty clearing station near the front line trenches in France. In the uncomplicated hemorrhage cases, an examination of the blood disclosed lowered alkaline reserve. In cases of acidosis and low blood pressure due to shock, hemorrhage, or infection with the gas bacillus, the pulse was rapid, but did not vary with the degree of acidosis. As the acidosis became more extreme, the respiratory rate increased, fatal cases showing true "air hunger" before death. As regards the relative reduction in alkaline reserve by the conditions of shock, hemorrhage, and infection with gas bacillus, Cannon's figures indicate the probability that uncomplicated hemorrhage is not followed by so great a reduction of alkaline reserve as is shock and infection with gas bacillus when the blood pressures are equally reduced.

Penfield[4] observed that acidosis developed gradually when the blood pressures of etherized dogs were brought to a low level by progressive hemorrhage, about 100 per cent of the total blood volume (calculated as 5 per cent of body weight) being removed. After varying lengths of time, the pressure was raised by infusion of different solutions. With one exception, death occurred only in those animals whose alkaline reserve was low.

Morriss[5] studied the effect of ether anesthesia on the alkaline reserve, using Van Slyke's method. He found a reduction in the reserve alkali in every case, but this reduction was not dependent upon the duration of ether anesthesia or upon the extent of the operation. He also found that the alkaline reserve might decrease notably when the patient lost relatively little blood, and on the other hand, might not change greatly when conspicuous hemorrhage occurred. He concluded that a notable drop might occur in the carbon dioxide-combining power of the plasma during the first half hour of anesthesia.

[3] Cannon, W. B., Fraser, J., and Hooper, A. N., *J. Am. Med. Assn.*, 1918, lxx, 526. Cannon, W. B., *ibid.*, 531.

[4] Penfield, W. G., *Am. J. Physiol.*, 1919, xlviii, 121.

[5] Morriss, W. H., *J. Am. Med. Assn.*, 1917, lxviii, 1391.

The Reaction Regulator Mechanism.

Dependent upon the proper hydrogen ion concentration of the blood are such vital processes as the control of the respiratory and vasomotor centers, enzyme action, the swelling of colloids, and cellular oxidation. Fortunately the organism has a protective mechanism which guards against the acids which are constantly formed in the animal organism by the normal metabolism of its tissue and the foods ingested. This mechanism may be regarded as consisting of several factors; first, the function of the buffer salts; second, the ability of the organism to excrete the weak acids (carbonic and phosphoric acids) set free by stronger acids; and third, the ability of the organism to produce ammonia at the expense of urea.

Van Slyke's method for the determination of alkaline reserve is based upon the principle that the power of the organism to protect itself against the encroachment of acids is proportional to the bicarbonate content of the arterial blood. In actual practice Van Slyke analyzes the plasma obtained from centrifugation of venous blood and reports the "alkaline reserve" in terms of volumes per cent of carbon dioxide chemically bound by 100 cc. of plasma. The possibility that HCl may pass from plasma to cells or *vice versa*, depending on the CO_2 tension, makes the precaution necessary that the blood should always be at definite CO_2 tension when centrifuged, as well as when analyzed. Van Slyke[2] and Austin and Jonas[6] carefully studied the effect on alkaline reserve of variations in the technique used in collecting the blood, and preparing the plasma for analysis.

The immediate cause and opportunity for this investigation were furnished by the plant for making serum to be used in vaccination against hog cholera, which is operated in connection with the College of Agriculture of the University of Wisconsin. In this plant large numbers of pigs are hyperimmunized against hog cholera. The animals are subsequently bled, the blood is defibrinized, and the serum used commercially. In this process, a certain routine, found by experience to be satisfactory and economical, has been established. Approximately 10 days after the animals have been hyperimmunized they are subjected to

[6] Austin, J. H., and Jonas, L., *Am. J. Med. Sc.*, 1917, cliii, 81.

tail bleeding, if they have not lost in weight. After an interval of 7 days the pig is subjected to a second tail bleeding if it has not lost in weight, and after 7 more days the pig is slaughtered.

EXPERIMENTAL.

In preliminary experiments, the blood was treated by what will hereafter be referred to in this paper as the "open vessel method" or Method I. The blood was allowed to flow directly from the tail into a small open Erlenmeyer flask which contained sufficient potassium oxalate to prevent clotting. Under the conditions of the experiment, the blood spurted rapidly from the artery directly into the flask, and so came into minimum contact with the tissues. No needle or other apparatus was required in collecting the sample. The flask was then rotated once or twice to insure the oxalate being dissolved in the blood, and was then carried to the laboratory for centrifugation with as little agitation as possible. The interval between collection and centrifugation was approximately ½ hour.

Standardization of Methods.

In order to discover the effect of this treatment on the alkaline reserve value, the following experiments were performed. Animals were kept at the laboratory in cages, and samples of blood were collected and treated under different conditions. In the first instance a stream of blood was allowed to spurt directly from the tail artery into a paraffined Erlenmeyer flask containing a small amount of potassium oxalate. The blood was centrifuged and the plasma analyzed by means of Van Slyke's technique.

This "open vessel method," when the blood was centrifuged and the plasma analyzed immediately, was very nearly comparable with Van Slyke's routine technique when he used a McRae needle for collection. By the open vessel method, it is true, the blood necessarily falls twice for a short distance through the air. This fact would seem to double the possibility for loss of carbon dioxide. So much less agitation was necessary, however, to insure thorough mixing of the oxalate with the blood in a receptacle like an Erlenmeyer flask that it seemed preferable to make the collections in such a flask rather than in a centrifuge tube.

This was particularly true in cases where the blood clotted rapidly, as after repeated hemorrhage. Because of the necessity, when working with a pig, of taking the blood samples from a cut tail, it was impossible to collect samples by Van Slyke's paraffin oil method; *i.e.*, without loss of some carbon dioxide.

Open Vessel Method.—Bloods collected by the open vessel method in the presence of sufficient potassium oxalate to prevent clotting were treated as follows.

I-a-1.—The blood was poured directly into centrifuge tubes, centrifuged, and the plasma analyzed at once (Curve I, Fig. 1).

I-a-2.—The blood was centrifuged as described under I-*a*-1. The clear plasma was pipetted into a paraffined weighing bottle, preserved in a refrigerator for 48 hours, and was then analyzed (Curve II, Fig. 1).

I-b.—The blood was allowed to remain in the Erlenmeyer flask in which it was collected for ½ hour. It was then centrifuged and the plasma analyzed immediately (Curve III, Fig. 1).

I-c.—The technique was the same as I-*b*, the time before centrifugation being extended to 2 hours (Curve IV, Fig. 1).

In the second instance an effort was made to approximate Van Slyke's paraffin oil method by allowing the blood to flow directly from the tail artery into a paraffined separatory funnel which had been previously filled with alveolar air. This technique was called the "separatory funnel method" or Method II. Duplicate collections of the blood analyzed by the open vessel method were made by the separatory funnel method and the samples so obtained were treated in various manners. Since the separatory funnel method was more laborious, and the results obtained were not duplicated so easily, this method proved to have no advantage over Method I, and was consequently abandoned. For the sake of brevity, the analyses by this method are not reported here.

Table I gives the results of the preliminary analyses. In all cases the figures represent the volumes per cent of CO_2 obtained from 1 cc. of plasma, and are the average of closely agreeing duplicates. After the first three series, all collections were made in paraffined vessels.

All the samples reported in the same vertical column of Table I were taken from the same animal, one sample immediately following another, and were then treated in the various manners described. The blood for the first five series of determinations was obtained from a female; for the second four series from a male.

Each weighed approximately 200 pounds. The extreme variations in the alkaline reserve values of the blood from the same animal on different days will be discussed in detail later.

The column headed "average difference" was computed as follows. The first method of determination (the open vessel method, centrifuged and analyzed immediately) was arbitrarily selected as the standard. If subsequent determinations were lower, the difference was indicated by a negative sign, and if

TABLE I.

Effect of Various Methods on Alkaline Reserve Values.

Method.	Alkaline reserve of blood from Pig 1.					Alkaline reserve of blood from Pig 2.				Average difference.
	June 27.	June 27.	June 28.	July 2	July 3.	July 5.	July 5.	July 6.	July 8.	
	a.m.	*p.m.*	*a.m.*	*a.m.*	*a.m.*	*a.m.*	*p.m.*	*a.m.*	*a.m.*	
I–*a*–1. Centrifuged and analyzed immediately.	49.4	56.7	25.8	53.1	58.4	51.3	29.1	48.3	54.0	±0
I–*a*–2. Centrifuged immediately; analyzed after 2 days.	46.4	53.5	29.5	52.8	59.0	50.8	27.9	48.4		−4.5
I-*b*. Centrifuged after ½ hour; then analyzed at once.	36.7	48.8	27.7	51.9	56.5	49.7	27.2	45.0	52.6	−3.3
I-*c*. Centrifuged after 2 hours; then analyzed at once.		49.2	29.6	51.9	54.1	46.5	26.3	44.2	50.3	−3.1

higher, by a positive sign. It will be seen that the average fall in alkaline reserve after intervals of ½ and 2 hours respectively is almost identical, and the maximum variation in the entire series between these two values is 3.2 volumes per cent.

The results obtained by the open vessel method are expressed graphically in Fig. 1. The alkaline reserve values are plotted on the vertical axis, and values in the same series (that is on the bloods collected at the same time, but subjected to different techniques) are given the same position on the horizontal axis.

Consequently, consistent results among the different techniques should yield parallel lines. That the lines to a certain extent approximate the parallel is an indication not only that the results can, within limits, be duplicated, but also that the time element is not an all-important factor. In other words, samples analyzed ½ hour or 2 hours after collection may not give identical values with the same samples analyzed immediately, but at least, corresponding time intervals yield samples that are, within limits, comparable. There is not extreme variation between short intervals, as, for instance, the ½ and 2 hour intervals.

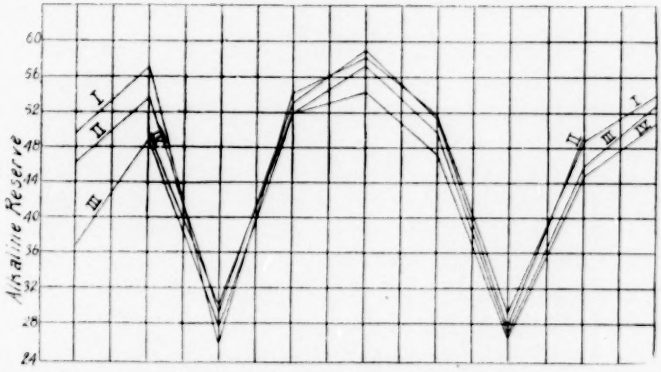

FIG. 1. Open vessel method. Illustrates the effect of different preliminary treatment on the alkaline reserve values of the same bloods. Alkaline reserve values are plotted on the ordinates and values obtained from the same collections, but analyzed by various techniques, are given the same position on the abscissæ. The general parallelism of the lines indicates that the results can be duplicated and that the various treatments yield consistent results.

Another attempt was made to find a technique which would be comparable with Van Slyke's method in which there was no loss of CO_2 during collection. Austin and Jonas[6] found that when they brought the whole blood into equilibrium with alveolar air immediately before centrifugation, their results were slightly higher than results obtained by Van Slyke's method, but were consistent with them. Since the details of Austin and Jonas' procedure could not be found, the following procedure was adopted. This technique will subsequently be referred to as the "alveolar air method" or Method III.

The blood was allowed to flow directly from the tail blood vessels into a paraffined separatory funnel of 300 cc. capacity, into which sufficient potassium oxalate had been introduced to prevent clotting. The funnel was then turned upside down once or twice to insure the oxalate being mixed with the blood. Just before the blood was centrifuged, alveolar air was blown into the funnel, and the funnel was then rotated end over end at the rate of one complete revolution per second for 60 seconds. Alveolar air was again blown into the funnel, and the process was repeated. In all, three such aspirations were made. Theoretically, if the composition of the alveolar air in the funnel were 6 per cent CO_2, then, during the three aspirations, 54 cc. of CO_2, measured at the same temperature and pressure, would have been introduced. The average difference (as will be shown later) between simultaneous determinations by Methods I and III is approximately 10 volumes per cent, or 0.10 cc. of CO_2 for every 1.0 cc. of plasma. It is not known how much CO_2 is absorbed by the corpuscles under these conditions. Furthermore, a liquid and a gas are in equilibrium not when their percentage compositions are the same but when their partial pressures are equal; *i.e.*, when the pressure of the gas on the liquid is equal to the tendency of the gas to leave the surface of the liquid. Since the actual mass of CO_2 absorbed by the blood from the alveolar air was such a small proportion of the mass of CO_2 actually introduced, and since the alveolar air was introduced in three separate portions, and each time was allowed to come into intimate diffusion relations with the blood, it seemed highly probable that the blood had absorbed as much CO_2 as it could be made to hold under these conditions, and that this technique was comparable to that of Austin and Jonas in which they brought the blood into equilibrium with alveolar air before centrifuging. The whole blood was then drawn off from the separatory funnel into a centrifuge tube under paraffin oil and was centrifuged under a layer of the oil. A great many determinations of alkaline reserve were made from samples of blood which were simultaneously collected, centrifuged, and analyzed, one sample being treated in the manner described for Method I and another in the manner described for Method III. In Tables II, III, and IV and Figs. 2 to 8 will be found the records of the determinations so made. In some cases (Figs. 2,

3, and 4, and Table II) a definite effort was made to ascertain the effect on the alkaline reserve values of allowing the blood to stand varying lengths of time before being centrifuged. The object of this experiment was to standardize the methods which might be used in subsequent experiments. In this work it was not the absolute alkaline reserve values of the blood as it existed

TABLE II.

Effect of Time before Centrifugation on Alkaline Reserve Values by Various Methods.

Animal No.	Time after collection before centrifugation.	Alkaline reserve.		Temperature.	Remarks.
		Method I.	Method III.		
	min.			°C.	
3	70	54.3	60.5	32	See Fig. 2.
	70	53.7	59.6		
	185	51.7	55.6		
	185	50.1	56.9		
	360	50.6	53.5		
	360	50.1	53.5		
4	55	48.1	60.6	26	See Fig. 3.
	85	48.1	58.6		
	115	48.1	60.0		
	145	48.6	58.1		
	175	48.2	58.3		
	305	48.0	56.4		
5	75	26.7	41.4	22	See Fig. 4.
	105	26.4	40.9		
	135	26.2	39.7		
	165	25.9			
	195	26.7	25.9		

in the animal body which were sought, but the relative values before and after hemorrhage.

It was noticed early in the work that fairly constant differences existed between the values obtained by Methods I and III for different sets of samples collected and analyzed under the same conditions. For instance, on one day, the average difference of six sets of samples analyzed by Methods I and III was 4.9, the maximum variation from this average figure being ± 1.6. On

another day, the average difference between the two methods run on the same number of samples was 8.2, the maximum variation from this figure being 1.9. Figs. 2 to 8 show that curves representing values obtained by the two methods for the most part run fairly parallel, each curve tending to afford evidence of the comparative accuracy of the data represented by the other. Furthermore, the curves plotted from data obtained by Method I are noticeably more regular, and hence are subject to fewer minor variations, than those plotted from data obtained by Method III. The degree of accuracy with which separate collections and centrifugations of samples could be duplicated by the two methods is indicated by the first series of data in Table II. An average of

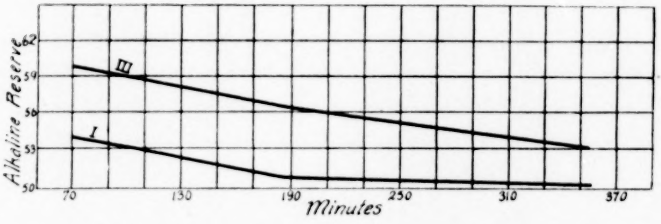

FIG. 2. On Curves I and III are plotted the alkaline reserve values obtained from samples collected at the same time, but allowed to stand definite periods of time before being centrifuged. Curve I represents values obtained by the open vessel method; Curve III, by the alveolar air method. Duplicate collections as well as duplicate analyses were made in each case, and average figures used. Temperature 32°C.

these duplicates was used in plotting Fig. 2. Although, as will be explained presently, Method III more nearly approaches an accurate picture of the true alkaline reserve values, results obtained by Method I are more easily duplicated, and apparently bear a more or less definite relation to the alkaline reserve values of the blood as it exists in the animal body.

A partial explanation for the variation in the relative values obtained by the two methods was found in the variation in temperature at the time when the samples were centrifuged. It happened that the data expressed in Fig. 2 were obtained on a summer day when the temperature was 32°C. The time in minutes after the samples were collected before they were centrifuged and

analyzed (or preserved in paraffined weighing bottles in an ice box) is plotted on the horizontal axis, the alkaline reserve values being plotted on the vertical axis. Although these data are very meager, they would seem to indicate that there was a small, though appreciable loss of CO_2 from the samples, even after the hour period, a point which was not illustrated in Fig. 1. To test further this possibility, two similar experiments were run, when the temperature was somewhat lower (Figs. 3 and 4). These results tend to uphold the conclusion that after the initial loss in CO_2 by a blood sample, which occurs during the first $\frac{1}{2}$ hour after the blood is drawn, there is very little subsequent loss during the following 2

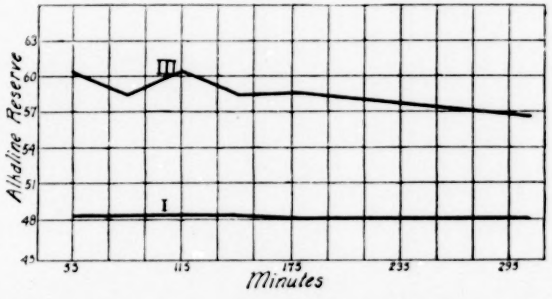

FIG. 3. Curve I represents alkaline reserve values obtained by the open vessel method on simultaneous collections which were allowed to stand varying lengths of time before centrifugation. Curve III represents values obtained on duplicate collections, treated by the alveolar air method. Temperature 26°C. In general, values obtained by Method III are higher than those obtained by Method I, but are consistent with them.

hours at ordinary temperatures; and that the values so obtained bear a more or less definite relation to those obtained when the blood is analyzed as soon as it is drawn. In only one case was a single value obtained which was entirely out of harmony with these ideas. This value is plotted at the last point in Curve III, Fig. 4. This plasma had been kept in an ice box for 4 days before it was analyzed. In general, the experience of the author entirely confirmed that of Van Slyke, inasmuch as plasma could be kept in an ice box for 7 days without appreciable loss in CO_2 content. The conclusion seems justifiable in this case that the plasma had undergone bacterial change or had become contaminated in some

other way. This contention is supported by another experiment. Two 15 cc. samples of blood (the alkaline reserve values of duplicate collections, by the open vessel method, were centrifuged and analyzed immediately, and were 53.8 and 58.4 respectively) were subjected for 15 minutes to the reduced pressure produced by a powerful vacuum pump. The blood was then centrifuged and analyzed, these values being 38.7 and 39.6 respectively. In these two cases, where the maximum loss of CO_2 might have been expected, the actual loss was 15.1 and 18.8 volumes per cent. Doubtless, then, the drop of 15.8 referred to above was not due to accidental loss of CO_2.

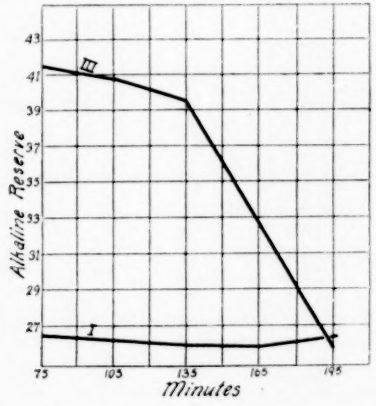

FIG. 4. Curve I represents values obtained by the open vessel method and Curve III by the alveolar air method, as in case of Figs. 2 and 3. Temperature 22°C. The plasma represented by the last point on Curve III had undoubtedly undergone bacterial change.

Although temperature is doubtless a contributing factor to the relative differences found in the analysis of duplicate samples by Methods I and III, examination of the curves indicates that, in general, larger differences were obtained when samples having comparatively high alkaline reserve values were analyzed. Fig. 8 illustrates this point. When the lowest values were reached at the 60 minute interval, the difference was 3 volumes per cent as compared with an initial difference of 15. If one had two solutions containing carbon dioxide in different proportions, and if one

manipulated these solutions in such a way that some carbon dioxide was lost, both solutions being treated in like manner, it seems reasonable to suppose that a larger mass of carbon dioxide would be lost by the solution containing the larger per cent of carbon dioxide. If one may reason by analogy, it seems logical that more carbon dioxide would be lost in the open vessel technique if the initial bicarbonate content were high than if it were low. Since loss of carbon dioxide is guarded against by Method III, greater differences between the two methods would be apparent when the initial bicarbonate content was comparatively high.

Influence of Hemorrhage on Alkaline Reserve.

The most surprising feature of the alkaline reserve values obtained in this preliminary work on methods was the fact that blood shed by the same animal varied tremendously on different days (Table I). The values recorded in the columns headed June 28 a.m. and July 5 p.m. were in each case obtained from the same animal as the values in the columns immediately preceding and following. The idea might be advanced that the drops in alkaline reserve were the result of loss of blood from a previous bleeding. This seems highly improbable, however, because in each bleeding in this series, not more than 100 cc. of blood were taken at a time. Furthermore, there was no relationship between the frequency of bleeding and the alkaline reserve values. As the experiment proceeded, it became evident that the high values were invariably associated with the bleedings which were accomplished without any struggle on the part of the animal before or at the time when the blood was drawn. The two extremely low values referred to were associated with violent struggling, and the intermediate values represented corresponding degrees of struggle. This point is further borne out by subsequent experiments. The data in Table III, which are taken from a series of tail bleedings, illustrate this point. In this table, a lower alkaline reserve value at the end of the bleeding is shown by a positive sign in the column headed "loss in alkaline reserve during bleeding," and *vice versa*. It is evident that, in those cases where little or no struggle took place during bleeding, the alkaline reserve values of the last 10 cc. of the large bleeding did not differ greatly from the values of the

first 10 cc. This fact is emphasized here in order that it may be borne in mind in connection with the data obtained in subsequent bleeding experiments.

In a preliminary experiment an attempt was made to follow the alkaline reserve values of a pig daily. Because of the difficulty in obtaining samples, and because the work of Milroy and others pointed to the fact that the drop in alkaline reserve caused by hemorrhage occurs soon after the blood is shed, no further attempts

TABLE III.

Typical Changes in Alkaline Reserve during the Process of Bleeding.

Animal No.	Weight.	Blood drawn.	Method.	Alkaline reserve.		Loss in alkaline reserve during bleeding.	Remarks.
				1st 10 cc. drawn.	Last 10 cc. drawn.		
	lbs.	*cc.*					
21	289	1,400	I	47.3	43.9	+3.4	Quiet throughout; bled freely.
21	289	1,400	III	55.9	56.9	−1.0	
7	245	1,000	I	50.4	51.2	−0.8	Quiet throughout; bled slowly.
52	272	1,000	I	51.2	48.9	+2.3	Quiet throughout; bled slowly.
18	210	1,300	I	61.6	59.7	+1.9	Quiet throughout; bled freely.
18	210	1,300	III	69.2	68.1	+1.1	
6	291	1,400	I	45.1	29.1	+16.0	Quiet before bleeding; struggled violently during bleeding; bled freely.
6	291	1,400	III	54.5	36.1	+18.4	

were made to collect daily samples. The procedure subsequently adopted was as follows. The animal was confined in a crate, and the tail was shaved. A small portion of the tail was then cut off, and the first few cc. of blood (about 5 cc.) were discarded. When the blood spurted from the artery in a good stream, two small samples (about 10 cc. each) were collected for analysis by Methods I and III. The results from these samples in each case were plotted as the first points on the curves. The cut tail was introduced into the vacuum flask and sufficient blood was drawn

to approximate 6 cc. per pound of body weight. Two more small samples were then taken as before, and the time of sampling was noted. The intervals between the samples at the beginning and at the end of the large bleeding indicated the duration of bleeding. From these data the approximate rate of bleeding could be calculated. Since the rate of bleeding varied consider-

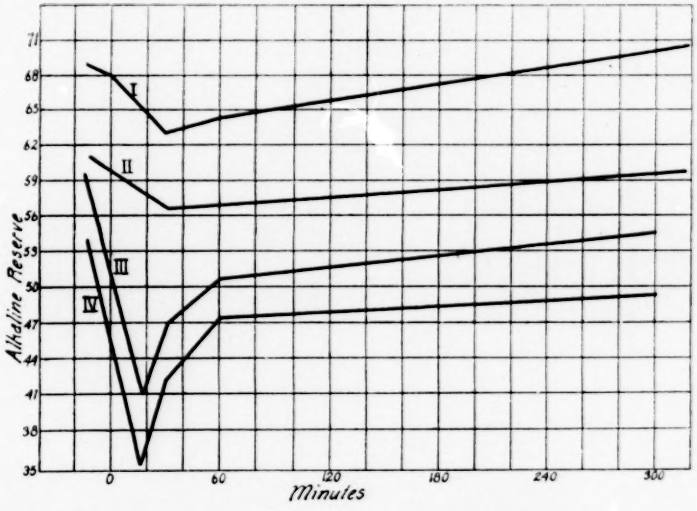

FIG. 5. Curve I (alveolar air method) and Curve II (open vessel method) illustrate the drop in alkaline reserve following the first hemorrhage of Sow 20. The time (abscissæ) is expressed in minutes before or after the large hemorrhage was completed; *i.e.*, the first point on the curves represents the alkaline reserve values of the first blood shed, the second point (at 0) the values of the last 10 cc. of the large bleeding. Curve III (alveolar air method) and Curve IV (open vessel method) represent a second hemorrhage of the same animal a week later (Table IV).

ably at different times, all subsequent samples were timed from the minute at which the large sample had been completely drawn. The animal was left in the crate, and the samples were taken at the intervals desired. Loss of blood between samples was prevented by tightly winding a rubber band around the end of the tail. At the end of the hour, the pig was taken from the crate, put into a pen, and was not disturbed until another sample was

TABLE IV.

Effect of Hemorrhage on Alkaline Reserve.

Animal No.	Description.	Weight.	Blood drawn.	Time of sampling.	Alkaline reserve. Method I.	Alkaline reserve. Method III.	Remarks.
		lbs.	*cc.*				
20	Not hyper-immune. ♀	210	1,260	First 10 cc. of big bleeding.	61.6	69.2	Bleeding lasted 8 min; very quiet throughout; no difficulty with respiration. (Fig. 5.)
				Last 10 " " " "	59.7	68.1	
				30 min. after end of "	56.5	63.8	
				60 " " " " "	57.4	64.9	
				317 " " " " "	60.6	70.7	
20 (7 days later.)	Not hyper-immune. ♀	234	1,400	First 10 cc. of big bleeding.	54.3	60.5	Struggled just when samples were taken at end of big bleeding; struggled considerably during first ½ hr. after bleeding; restless during second ½ hr.; respiration normal throughout; necessary to cut the tail again to collect the last sample; bleeding lasted 14 min. (Fig. 5.)
				Last 10 " " " "	46.6	50.8	
				15 min. after end of "	35.3	41.0	
				30 " " " " "	42.3	47.0	
				60 " " " " "	47.6	50.9	
				300 " " " " "	49.5	55.1	
21	Hyper-immune. ♀	299	1,333	First 10 cc. of big bleeding.		53.0	Bleeding lasted 8 min.; struggled when samples were taken; otherwise quiet throughout; respiration normal. (Fig. 6.)
				Last 10 " " " "	37.9	47.0	
				15 min. after end of "	33.7	44.5	
				30 " " " " "	30.9	37.7	
				60 " " " " "	31.0	39.0	
22	Hyper-immune. ♀	202	1,260	First 10 cc. of big bleeding.	48.1	60.6	Bleeding lasted 23 min.; poor bleeder; tail was cut 3 times during big bleeding; fairly quiet throughout, except when the last samples were taken. (Fig. 7.)
				Last 10 " " " "	41.9	51.2	
				15 min. after end of "	41.9	50.7	
				30 " " " " "	43.0	49.5	
				60 " " " " "	48.0	60.5	
				330 " " " " "	46.1	52.5	

23	Hyper-immune. ♂	216	1,300	First 10 cc. of big bleeding.	26.7	41.4	Bleeding lasted 37 min.; blood unusually dark; struggled all the time while in the crate (1½ hrs.); all analyses run in duplicate; animal survived. (Fig. 8.)
				Last 10 " " " "	12.3	23.4	
				15 min. after end of "	12.3	23.3	
				30 " " " " "	12.1	14.0	
				60 " " " " "	2.0*	5.0*	
				287 " " " " "	21.7	26.2	

* These values were obtained by plotting the values given in Van Slyke's tables and reading these values from the curve.

taken in the same way at the end of an approximate 5 hour interval. In most cases it was a simple matter to obtain the 5 hour sample. Occasionally it became necessary to cut the tail again. In such cases the fact has been noted.

In the first experiment of this kind, samples were collected for analysis by both methods when 30 and 60 minutes had elapsed after the big sample had been drawn. In this case, as is shown by Curves I and II, Fig. 5, the drop in alkaline reserve was comparatively small, the lowest point being reached at the 30 minute interval. The 5 hour sample showed that the alkaline reserve

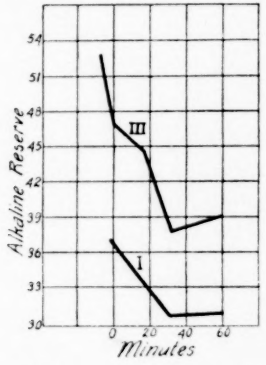

FIG. 6. Curve III (alveolar air method) and Curve I (open vessel method) illustrate the performance of Sow 21, a hyperimmune, during a second hemorrhage (Table IV).

was very nearly at its original value. Since the drop in alkaline reserve was so small (about 5 volumes per cent) it seemed likely that the low point on the curve had been missed. In the subsequent experiments, samples were taken 15 minutes after the end of the large bleeding also.

Curves III and IV, Fig. 5, show the performance of the same animal 7 days later, the total quantity of blood drawn being slightly larger than that taken the previous week. In this case, the alkaline reserve was consistently lower throughout. The drop in alkaline reserve on that day, due, presumably, to loss of blood, was much greater; the lowest point being reached at the end of 15 minutes.

Considerable data of this nature were accumulated, without any attempt at further analysis of these samples. The data are summarized in Table IV, and expressed graphically in Figs. 5, 6, 7, and 8. Further information concerning the drop in alkaline reserve associated with loss of blood is furnished by subsequent more elaborate experiments.

These experiments were of particular interest inasmuch as they indicated that there was great variation in the drop in alkaline reserve due to hemorrhage. It is a well recognized fact that after loss of blood, the blood volume is rapidly restored, and an efficient

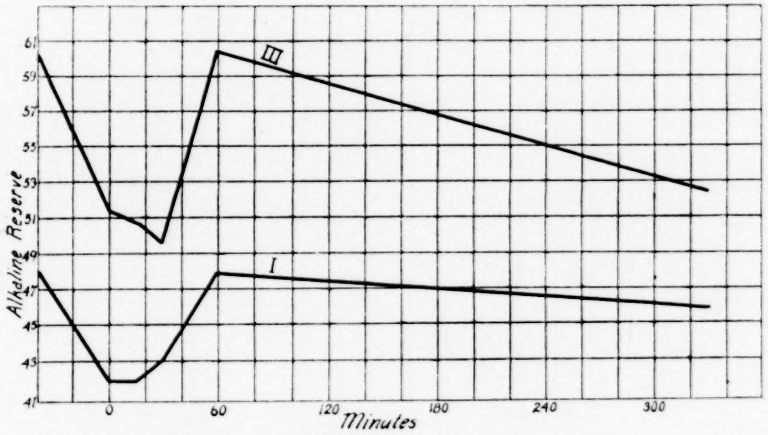

FIG. 7. Curve III (alveolar air method) and Curve I (open vessel method) illustrate the effect of a first hemorrhage on Sow 22 (Table IV).

blood pressure consequently maintained, by a dilution of the blood with the tissue fluids. Consequently, a drop in the total nitrogen content of the blood might be expected.

Two pigs (females, Nos. 7 and 8), which were not hyperimmune, were bled in the usual manner, the alkaline reserve values (obtained by the open vessel method) being recorded in Tables V and VI, and plotted in Figs. 9 and 10. Three successive bleedings were made on these two animals, at weekly intervals. The total nitrogen of the blood was determined by the Kjeldahl method, the samples being weighed in all cases. The results are averages of closely agreeing triplicate determinations. The usual drop in

percentage of nitrogen in 5 hours, due to hemorrhage of this magnitude, was 0.3 to 0.4 per cent. No explanation is advanced for the unusual drop of 1.01 per cent found in the second bleeding (Table V). More data on this point will be presented later. This experiment illustrates the individual reaction of animals subjected to the same treatment. The animals described in Tables V and VI were similar in size, weight, and sex, but one

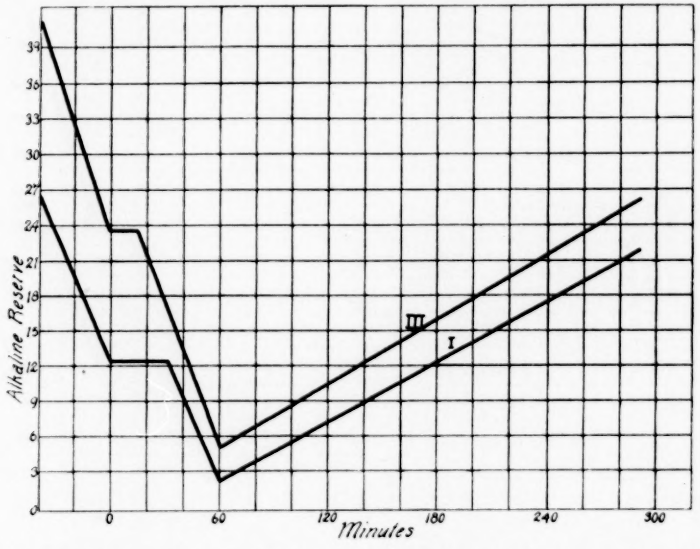

FIG. 8. Curve III (alveolar air method) and Curve I (open vessel method) illustrate the effect of a first hemorrhage on No. 23, a black male. The alkaline reserve values reached the lowest point encountered in this work. This experiment shows the combined effect of violent struggle plus hemorrhage in lowering alkaline reserve (Table IV).

suffered a greater loss in reserve alkali on bleeding than the other. No. 8 (Table VI) developed an acidosis, due to violent struggle and loss of blood, which was less severe, in terms of actual alkaline reserve values, than that developed by No. 7 (Table V, second bleeding) when she was quiet throughout. The drop in alkaline reserve was the same in both cases. It is evident from data gathered by the repeated bleeding of the same animal that the loss of reserve alkali immediately following hemorrhage was not

TABLE V.

Effect of Repeated Hemorrhage on Blood of Pig 7.

Date.	Weight.	Blood drawn.	Time of sampling.	Alkaline reserve.		Total nitrogen.		Remarks.
				Volumes per cent.	Maximum drop.	Per cent.	Drop.	
1918	*lbs.*	*cc.*						
Oct. 4	270	1,625	First 10 cc. of big bleeding.	47.3		3.16		Fairly quiet throughout. (Fig. 9, Curve I.)
1st bleeding.			Last 10 " " " "	44.5				
			15 min. after end of "	44.5				
			30 " " " " "	42.6				
			60 " " " " "	39.0	8.3			
			325 " " " " "	44.6		2.85	0.31	
Oct. 11	280	1,680	First 10 cc. of big bleeding.	42.2		2.90		Fairly quiet throughout. (Fig. 9, Curve II.)
2nd bleeding.			Last 10 " " " "	43.0				
			15 min. after end of "	34.6				
			30 " " " " "	26.6	16.4			
			60 " " " " "	34.0				
			297 " " " " "	36.4		1.89	1.01	
Oct. 18	276	1,650	First 10 cc. of big bleeding.	63.0		2.72		Fairly quiet throughout. (Fig. 9, Curve III.)
3rd bleeding.			Last 10 " " " "	62.5				
			15 min. after end of "	54.8				
			30 " " " " "	51.2	11.8			
			60 " " " " "	54.6				
			300 " " " " "	54.3		2.44	0.28	

TABLE VI.

Effect of Repeated Hemorrhage on Blood of Pig 8.

Date.	Weight.	Blood drawn.	Time of sampling.	Alkaline reserve.		Total nitrogen.		Remarks.
				Volumes per cent.	Maximum drop.	Per cent.	Drop.	
1918	*lbs.*	*cc.*						
Oct. 4	270	1,625	First 10 cc. of big bleeding.	56.7		2.89		Struggled considerably. (Fig. 10, Curve I.)
1st bleeding.			Last 10 " " " "	47.3				
			15 min. after end of "	47.3				
			30 " " " " "	40.4	16.3			
			60 " " " " "	44.6				
			288 " " " " "	52.5		2.59	0.30	
Oct. 11	298	1,790	First 10 cc. of big bleeding.	47.5		2.74		Uneasy during bleeding; then fairly quiet. (Fig. 10, Curve II.)
2nd bleeding.			Last 10 " " " "	44.8				
			15 min. after end of "	43.9	3.6			
			30 " " " " "	46.1				
			60 " " " " "					
			260 " " " " "	46.5		2.33	0.41	
Oct. 18	276	1,650	First 10 cc. of big bleeding.	61.1		2.78		Fairly quiet throughout. (Fig. 10, Curve III.)
3rd bleeding.			Last 10 " " " "	61.1				
			15 min. after end of "	56.4	4.7			
			30 " " " " "	58.3				
			60 " " " " "	58.3				
			283 " " " " "	57.0		2.31	0.47	

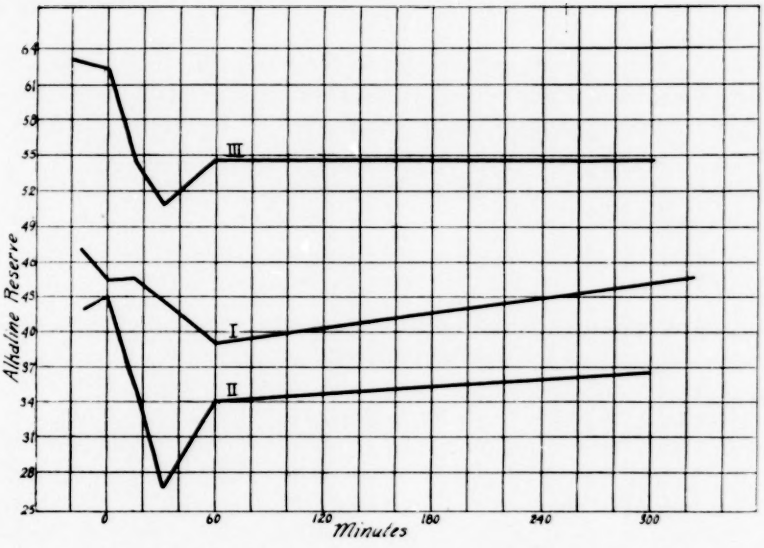

FIG. 9. Curves I, II, and III (open vessel method) illustrate the effect of a first, second, and third hemorrhage on a female, not hyperimmune (Table V).

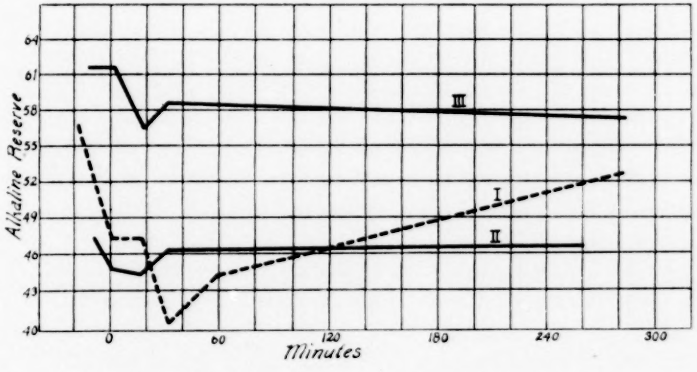

FIG. 10. Curves I, II, and III (open vessel method) illustrate the effect of a first, second, and third hemorrhage on a female, not hyperimmune (Table VI).

the only variation of alkaline reserve to which the animal was subject.

In many instances, the value obtained from the 5 hour sample was almost identical with that obtained from analysis of the first blood shed. The initial value obtained at the next series of bleedings, after the interval of 5 or 7 days, might be higher or lower. These animals were kept under ordinary farm conditions, and received a mixed diet.

Influence of Hemorrhage on Other Constituents.

These striking changes in the total nitrogen content of the blood made a study of the nitrogen distribution desirable.

Taylor and Lewis,[7] working with dogs, found, when large quantities of blood were withdrawn hourly under continuous ether anesthesia, and the blood volume so lost was compensated by the injection of Ringer's solution, that the blood serum showed the following changes. (*a*) A progressive decrease in the total nitrogen and total protein of the serum was less than the theoretical dilution of the blood would account for. The authors found no evidence, however, for the assumption of any synthesis of serum globulin or serum albumin during the time of the experiments. (*b*) They also found such a large progressive rise in non-protein nitrogen of the serum that they concluded that the increase could not have been due to any mere washing out of the tissues. Urea and amino nitrogen were also found to be increased with bleeding. They said in conclusion, "It does not seem possible to resist the conclusion that the increase of non-protein nitrogen has been the result of an active process on the part of the tissues, due either to a setting free of stored amino-acids or to amino-acids derived from hydrolysis of tissue (cellular) protein or serum protein."

In the next experiment Pig 9 was bled seven times, the interval between bleedings being 5 days (with one exception). The alkaline reserve samples were collected at the usual intervals, whenever it seemed advisable to do so. Toward the end of the experiment, the blood clotted so rapidly and persistently that samples could not be obtained without cutting the tail. Non-

[7] Taylor, A. E., and Lewis, H. B., *J. Biol. Chem.*, 1915, xxii, 71.

protein nitrogen was determined by the trichloracetic acid modification of the Folin and Denis[8] method. The ammonia was distilled according to the method of Bock and Benedict,[9] and the distillate was Nesslerized. Urea was determined by Van Slyke and Cullen's[10] modification of Marshall's urease method, the ammonia being Nesslerized according to the suggestion of Rose and Coleman.[11] Chlorides were determined by the method of McLean and Van Slyke,[12] the proteins being precipitated by means of copper sulfate in alkaline solution, according to the suggestion

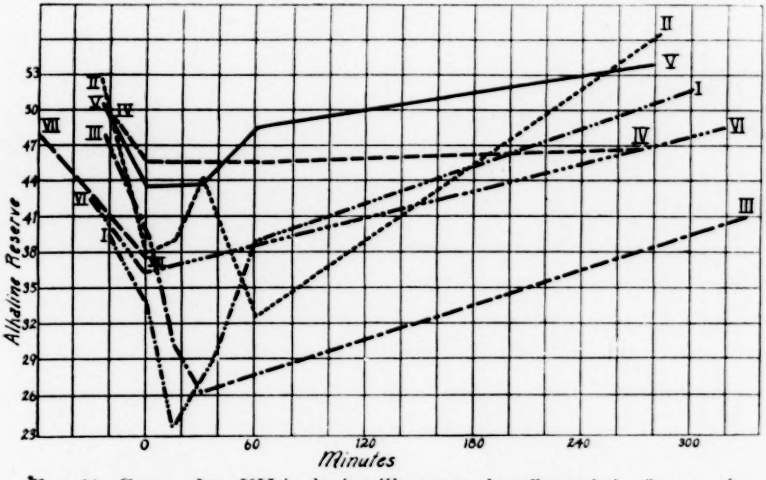

FIG. 11. Curves I to VII inclusive illustrate the effect of the first to the seventh bleeding of a female whose diet was limited to corn and tap water (Table VII). All determinations were made by the open vessel method.

of Harding and Mason.[13] All the determinations were run on whole blood, both on the first blood shed and also on the 5 hour samples. The results are summarized in Table VII and Fig. 11.

The diet of this animal was restricted to corn and tap water,

[8] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.
[9] Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 47.
[10] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.
[11] Rose, A. R., and Coleman, K. R., *Biochem. Bull.*, 1914, iii, 411.
[12] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.
[13] Harding, V. J., and Mason, E. H., *J. Biol. Chem.*, 1917, xxxi, 55.

TABLE VII.

Effect of Hemorrhage on Blood of Pig 9; Not Hyperimmune; Diet Limited to Corn and Water.

Date.	Weight.	Blood taken.	Time of sampling.	Alkaline reserve.		Total nitrogen.		Non-protein nitrogen.		Urea nitrogen.		Chlorides as NaCl.		Remarks.
				Volumes per cent.	Maximum drop.	Per cent.	Difference.	Per 100 gm.	Difference.	Per 100 gm.	Difference.	Per cent.	Difference.	
1918	*lbs.*	*cc.*					*mg.*	*mg.*	*mg.*	*mg.*	*mg.*			
Nov. 5	240	1,550	Start of bleeding.	39.6		3.16		27				0.69		Uneasy toward end of bleeding, but did not struggle; fairly quiet after big bleeding; taken from crate at end of 1 hr. (Fig. 11.)
1st bleeding, 18 min.			End " "	34.0										
			15 min. after end.	23.7	15.9									
			30 " " "	26.0										
			60 " " "	38.7										
			300 " " "	51.7		2.72	−0.44	26	−1			0.75	+0.06	
Nov. 10	258	1,600	Start of bleeding.	52.7		2.57		29				0.74		Uneasy during bleeding; fairly quiet after; apparently exhausted when taken from crate at end of 1 hr.
2nd bleeding, 27 min.			End " "	37.8										
			15 min. after end.	38.7										
			30 " " "	44.0										
			60 " " "	32.4	20.3									
			285 " " "	56.4		2.35	−0.17	29	±0			0.76	+0.02	
Nov. 16	261	1,730	Start of bleeding.	48.0		2.59		28		9		0.76		Very restless during bleeding; squealed vociferously; breathed heavily; respiration 160 per min. at end of ½ hr. (normal = 25-30); typical air hunger; tail would not bleed at end of 1 hr.; respiration 60; apparently normal after 5 hrs., respiration 40.
3rd bleeding, 24 min.			End " "	39.3										
			15 min. after end.	30.6										
			30 " " "	26.6	21.4									
			60 " " "											
			330 " " "	41.8		2.32	−0.27	34	+6	12	+3	0.79	0.03	

Nov. 21 4th bleeding, 25 min.	257	770	Start of bleeding.	50.4		2.54		31		13	0.73		Did not take full amount because of partial collapse of animal at previous bleeding; fairly quiet throughout; taken from the crate between sampling.
			End " "	45.8	4.6								
			30 min. after end.	45.8									
			60 " " "	45.8									
			270 " " "	46.7		2.49	−0.05	32	+1	13	±00.69	−0.04	
Nov. 27 5th bleeding, 25 min.	256	1,470	Start of bleeding.	50.4		2.68		24		8	0.73		Fairly quiet throughout; seemed to stand bleeding well; taken out of crate between sampling.
			End " "	43.7	4.7								
			30 min. after end.	43.7									
			60 " " "	48.5									
			275 " " "	54.0		2.45	−0.23	28	+4	14	+60.72	−0.01	
Dec. 2 6th bleeding, 32 min.	256	1,400	Start of bleeding.	42.4		2.87		29		7	0.74		Extremely difficult to get samples, because of rapid clotting; pig nervous, but did not struggle.
			End " "	36.4	6.0								
			315 min. after end.	48.5		2.32	−0.55	32	+3	9	+20.71	−0.03	
Dec. 7 7th bleeding, 60 min.	255	1,500	Start of bleeding.	47.3		2.74		27		7	0.74		Almost impossible to get samples because of rapid clotting; tail cut again to get last sample; pig apparently in good condition.
			End " "	37.3	10.0								

no salt being added to the ration. It is a recognized fact from the extensive work of Kerr, Hurwitz, and Whipple[14] that diet plays an important part in the rate of regeneration of blood. Little work has been done, however, on the effect of diet on the chemical composition of the blood. The aim of this experiment was to investigate the ability of the animal to make good the losses in alkaline reserve, due to repeated severe hemorrhages on a poor and incomplete diet which was known to be acid-producing, without the addition of salts. In this experiment no records were kept of the daily intake, and the excreta were not analyzed.

The losses in alkaline reserve were much greater the first three times the animal was bled than they were subsequently, although the data on the last bleedings are not so extensive as might be desired, due to the great difficulty in obtaining samples. When a good arterial stream could not be obtained, the samples were not collected. Apparently the alkaline reserve value of the first blood shed on any day, under the conditions of these experiments, bears no relation to such factors as the number of times the animal has been bled previously, or the amount of blood taken at previous bleedings. Whether the variations in alkaline reserve from week to week are entirely due to the experimental procedure, or are influenced by other factors to some extent is difficult to say. It happens that the initial alkaline reserve on the 1st day when the animal was bled is the lowest initial value obtained, and that the initial value on the last day is very nearly as high as any obtained. It appears, then, that this animal was capable of maintaining an efficient alkaline reserve over a period of 5 weeks on a diet which was highly acid in character, even though a total volume of blood amounting to 10 liters was withdrawn from the system during this time. So far as could be ascertained no figures obtained by recent methods are available concerning the total amount of blood in the pig. If, however, one-twelfth of the body weight is assumed to be blood, then the theoretical volume of blood in the animal at the beginning of the experiment was approximately 9 liters. Since considerably more than the probable initial blood volume was withdrawn during this experiment, it is evident that a tremendous stimulus to metabolism was afforded.

In this connection, it is of interest to note the total nitrogen content of the blood at the various stages, and the distribution

[14] Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918–19, xlvii, 356, 370.

of the nitrogen, as far as data are available. Drops in the per cent of nitrogen during the first 5 hours after bleeding are of the same order of magnitude as those reported in the previous experiment. In this case, however, there was a further drop in nitrogen content after the first bleeding. The intervals between bleedings were shorter in this experiment than in the preceding experiment. Beginning with the fourth bleeding, it is interesting to watch the total nitrogen rise. The animal is evidently succeeding in restoring some of the lost protein of the blood, even under these conditions of repeated hemorrhage, until finally, with the seventh bleeding, the figure falls slightly. That the protein of the blood was actually being increased is evidenced by the data on the non-protein nitrogen and urea nitrogen content of the blood. Although in some cases these values were higher 5 hours after bleeding than at the beginning of bleeding, there seemed to be no great tendency for these forms of nitrogenous bodies to accumulate in the blood to any significant extent. It is to be expected that in the mobilization of nitrogenous compounds necessitated by the loss of blood, unavailable portions of nitrogenous compounds would have to be excreted; and hence that the normal products of protein metabolism would accumulate temporarily in the blood. Of course, the small rise in non-protein nitrogen and urea nitrogen indicated by analysis in reality represents a very much larger rise in these compounds when the conditions existing in the body are remembered. The blood has been largely diluted with tissue fluids and the total mass of actively functioning tissue (the blood) has been materially reduced. In some cases, a decrease of these bodies, as shown by analysis, might mean an actual increase in their production. The fourth bleeding, where a comparatively small volume of blood was withdrawn, is a case in point. Although no salts were added to this diet which is known to be poor in chlorides, the changes in the percentage of chlorides were too small to be significant.

DISCUSSION.

There was a great variation in the reaction of different animals to hemorrhages of the same relative magnitude, and in the reaction of the same individual at different times. When the

same individual was bled a number of times, in all cases in which the animal struggled the alkaline reserve values dropped lower than in those cases in which the animal remained quiet. This fact was so universal that it seems safe to conclude that, other factors being equal, the drop in alkaline reserve in pigs, following hemorrhages of this magnitude, varies directly with the amount of struggling. In many cases, bleeding was accomplished without any apparent struggle. In such cases the drop in alkaline reserve was always small. Doubtless, if more blood had been drawn the drop in alkaline reserve might have been considerably greater even though the animal remained perfectly quiet. Since larger hemorrhages were not attempted, no definite information can be reported on this point. It is true that there was considerable variation in the amount of blood drawn at different times, but there were so many other factors which influenced the alkaline reserve values that differences in these values could not be attributed entirely to differences in the volume of blood drawn. Table VII offers a case in point. In the case of the fourth bleeding, when a small volume of blood was removed, there was a small drop in alkaline reserve; but in the next bleeding, where almost twice the volume of blood was removed, the same drop in alkaline reserve was obtained. In the third bleeding when an unusually large volume was removed, there was a large drop in alkaline reserve, but this experiment was complicated by struggling. In general, when comparatively large volumes of blood were removed, there was a large drop in alkaline reserve, but in such cases the bleeding was almost invariably associated with struggle and restlessness. How much of the acidosis is attributable to actual loss of blood and how much to the struggling is difficult to say, the tendency to struggle doubtless being the direct result of the loss of blood.

The statement frequently made in the literature that hemorrhage is accompanied by loss in reserve alkali or by a condition of acidosis, has been based upon experiments, as far as the author has been able to ascertain, in which the experimental animal has been subjected to anesthesia. Since anesthesia is known to lower the alkaline reserve of an animal,[5] the exact effect of the loss of blood is not clear. In the experiments described above this complicating factor has been eliminated. It is true that another

factor, struggle, has been introduced. The results obtained from the many hemorrhages which were successfully carried out without any apparent struggle afford evidence of the effect of loss of blood uncomplicated by anesthesia or struggle. The cases in which struggle took place are of particular interest inasmuch as more or less struggle usually accompanies the bleeding process. Furthermore, loss of blood in general is apt to be accompanied by struggle and nervous excitement.

Milroy's conclusion that there must have been very rapid compensatory passage of the tissue fluid into the circulation, the fluid first entering the circulation being extremely poor in reserve alkali, is not confirmed by these experiments in which the pig was the experimental animal and the Van Slyke apparatus was the means of measuring reserve alkali. In many cases the drop in alkaline reserve was slight. It is true that the hemorrhages which Milroy described were much more severe; but it seems reasonable to suppose that if the tissue fluids were poor in reserve alkali there would have been greater drops in alkaline reserve in many instances than were actually found.

The fact that one animal (Table VII and Fig. 11) when bled seven times at intervals of 5 days was able to maintain an efficient alkaline reserve throughout the experiment even though her diet was restricted to corn and water affords further evidence of the fact that acidosis is not the limiting factor which must be considered in the treatment of an animal which has lost a moderate amount of blood. It is true that diet undoubtedly is an extremely important factor in the rate of regeneration of blood.[14]

The rise in urea and non-protein nitrogen in the blood after bleeding might be caused by the breaking down of body proteins in an effort to replace the lost circulating protein. The urea might function also in maintaining normal osmotic relations.

CONCLUSIONS.

1. If the blood is allowed to flow directly from an artery into a paraffined vessel containing potassium oxalate, and if the blood so obtained is allowed to stand a definite time ($\frac{1}{2}$ to 2 hours)

before centrifugation, the alkaline reserve values obtained by analysis of the plasma by the Van Slyke method bear a fairly definite relation to the alkaline reserve of the blood as it exists in the body. These results can be duplicated with satisfactory accuracy.

2. Alkaline reserve values obtained in this manner are lower than values obtained when the blood is brought to a definite CO_2 tension immediately after centrifugation, but, under definite conditions, are consistent with them.

3. When pigs were subjected to hemorrhages amounting to approximately 1.3 per cent of body weight, a study of the alkaline reserves of these animals immediately after the hemorrhages resulted in the following observations.

(*a*) Hemorrhages of this magnitude were usually accompanied by a somewhat lowered alkaline reserve during the first few hours after bleeding.

(*b*) When the animal remained perfectly quiet throughout the experiment, the drop in alkaline reserve was invariably small.

(*c*) If the animal struggled, the drop in alkaline reserve was much greater, this being noticeable soon after struggling took place.

(*d*) When the animal remained quiet, the maximum drop in alkaline reserve was reached within $\frac{1}{2}$ hour after the bleeding was completed. At the end of 5 hours, and often sooner, the alkaline reserve was near its original value.

(*e*) There was considerable variation in the reaction of different individuals.

(*f*) When the diet of one animal was restricted to corn and water and the animal was bled seven times at intervals of 5 days, the alkaline reserve value of the first blood shed on any day bore no relation to such factors as the number of times the animal had been bled previously, or the amount of blood (within the limits of these experiments) taken at previous bleedings.

4. The total nitrogen content of the blood always fell immediately after hemorrhage. There was a distinct tendency for the urea nitrogen and the non-protein nitrogen to rise. Although one animal was bled seven times while restricted to a diet of corn and water, the percentage of chlorides in the blood remained constant.

5. On an inadequate diet (corn and water) under conditions of repeated hemorrhage there was a distinct tendency toward regeneration of blood proteins.

Grateful acknowledgment is made of the valuable criticism and kindly cooperation of Professor Steenbock and Dr. Beach of the College of Agriculture, throughout the course of these experiments.

STUDIES OF BLOOD REGENERATION.*

II. EFFECT OF HEMORRHAGE ON NITROGEN METABOLISM.

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(Received for publication, August 11, 1919.)

INTRODUCTION.

The changes in the chemical composition of the blood of the pig under conditions of repeated hemorrhage, reported in the previous paper[1] are of interest in the light of investigations of the effect of hemorrhage and acidosis on metabolism. Hawk and Gies[2] concluded that in dogs in nitrogen equilibrium hemorrhage stimulated nitrogen excretion. Kerr and his coworkers[3] found that after plasmapharesis "The basal nitrogen metabolism shows no constant variation under these experimental conditions, with the exception of a primary rise in nitrogen elimination which occurs in the few days following the shock of the plasmapharesis." Haskins[4] found a decided rise in the amount of total nitrogen excreted on the 2 days following hemorrhage.

EXPERIMENTAL.

Pig A, a female weighing 330 pounds, was confined in a large metabolism cage, and was fed 5 pounds of corn (maize meal) each day, and 4 liters of distilled water. (Later 5 liters of water

* The work described in this article forms part of a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Wisconsin.

[1] Buell, M. V., *J. Biol. Chem.*, 1919, xl, 29.

[2] Hawk, P. B., and Gies, W. J., *Am. J. Physiol.*, 1904, xi, 171.

[3] Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918–19, xlvii, 356.

[4] Haskins, H. D., *J. Biol. Chem.*, 1907, iii, 321.

TABLE I.

Effect of Repeated Hemorrhage on Urine of Pig A.

Date.	Volume.	Specific gravity.	Reaction.	Total nitrogen.	Uric acid.	Uric acid N from total N.	Creatinine.	Creatinine N from total N.	Creatine.	Creatine N from total N.	Ammonia N.	Ammonia N from total N.	Urea N.	Urea N from total N.	P_2O_5	$\frac{P_2O_5}{N}$
1919	*cc.*		*pH*	*gm.*	*mg.*	*per cent*	*gm.*	*per cent*	*mg.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		
Jan. 11	820	1.037	5.8	19.3	309	0.48	1.56	2.17	322	0.52	2.22	11.5	13.4	69.5	4.18	0.22
" 12	1,580		5.8	16.1	311	0.58	1.64	2.74	281	0.54	3.07	19.0	11.3	70.0	4.18	0.26
" 13	2,525	1.013	6.8	16.6	282	0.51	1.41	2.29	363	0.68	2.75	16.5	11.8	70.9	2.63	0.16
" 14	2,300	1.011	6.0	14.8	308	0.62	1.41	2.55	314	0.66	2.12	14.6	10.5	70.8	3.91	0.26
" 15	2,675	1.010	6.0	17.4	366	0.63	1.69	3.00	372	0.66	2.59	14.8	12.3	70.7	4.05	0.23
" 16	2,200	1.006	6.6	10.6	243	0.69	1.13	2.88	268	0.79	1.45	13.7	7.5	71.4	2.35	0.22
" 17	3,310	1.007	5.6	25.5	418	0.49	2.45	2.58	439	0.54	3.92	15.4	16.4	64.4	6.27	0.25
" 18	1,375	1.020	5.9	19.3	397	0.62	1.64	2.29	657	1.06	3.98	20.6	13.7	71.2	5.40	0.28
" 19	2,350	1.014	6.0	22.7	329	0.43	1.72	2.04	831	1.14	3.32	14.6	15.8	69.7	5.29	0.23
" 20	2,525	1.012	6.6	24.7	303	0.37	2.16	2.35	692	0.87	4.03	16.3	13.9	56.4	6.47	0.26
" 21	1,415	1.014	6.6	14.7	271	0.55	1.58	2.88	619	1.31	2.78	18.8	8.9	60.2	3.73	0.25
" 22	1,000	1.010	6.4	7.9			0.85	2.91	188	0.74					1.79	0.23
" 23	2,300	1.023	6.4	26.4	419	0.48	2.83	2.89	278	0.33			16.2	61.7	9.85	0.37
" 24	1,150	1.011	5.8	9.1	128	0.42	0.87	2.51	121	0.41	1.34	14.6	7.5	82.5	2.42	0.26
" 25	1,050	1.018	5.8	14.6	256	0.52	1.36	2.50	481	1.02	1.95	13.3	10.2	69.9	3.38	0.23
" 26	3,200	1.010	5.6	25.3	425	0.50	2.51	2.67	810	1.00	3.13	12.3	18.2	71.9	6.44	0.25
" 27	780	1.013	5.6	7.7	112	0.44	0.80	2.82	294	1.19	0.98	12.8	5.3	69.4	1.67	0.22
" 28	3,250	1.013	6.6	23.6	406	0.51	2.55	2.90	1,121	1.48	4.71	19.9	14.3	60.7	8.61	0.36
" 29	2,020	1.012	5.6	13.6	263	0.46	1.46	2.87	428	0.98	1.85	13.5	9.5	66.8	6.33	0.46
" 30	2,000	1.008	5.6	10.5	188	0.53	1.21	3.08	237	0.70	1.43	13.5	7.3	69.0	4.75	0.45
" 31	2,600	1.015	6.8	14.9	278	0.57	1.48	2.67	270	0.45			5.2	35.4	6.92	0.46

Feb.	1	1,750	1.017	5.8	20.3	348	0.51	1.81	2.40	825	1.26	3.95	19.4	10.7	52.8	5.20	0.25
"	2	2,500	1.010	5.9	14.6	188	0.38	1.41	2.60	572	1.22	2.00	13.7	10.5	71.8	4.26	0.29
"	3	2,500	1.017	5.6	29.0	441	0.46	2.58	2.40	1,513	1.63	7.13	24.6	16.1	55.5	10.83	0.37
"	4	2,700	1.016	5.6	21.7	377	0.52	1.97	2.44	923	1.32	2.82	13.0	15.4	70.9	7.16	0.33
"	5	1,950	1.010	5.6	11.7	203	0.52	1.05	2.41	435	1.15	1.49	12.7	8.7	74.1	4.71	0.40
"	6	2,120	1.017	5.6	19.7	356	0.54	1.82	2.48	542	0.85	5.50	27.9	10.5	53.2	7.06	0.36
"	7	3,500	1.012	5.6	29.3	464	0.47	2.77	2.54	956	1.01	6.87	23.4	17.1	58.3	10.29	0.35
"	8	1,475	1.020	5.8	20.1	455	0.68	1.58	2.10	1,053	1.63	1.79	8.9	14.4	71.5	3.92	0.19
"	9	2,550	1.018	6.1	10.6	177	0.50	1.27	3.22	537	1.57	1.94	18.2	12.3		3.46	0.33
"	10	3.000		6.4	29.3	482	0.49	2.50	2.29	1,018	1.08	5.22	17.7	19.0	65.0	7.52	0.26
"	11	2,450	1.009	6.0	12.7	223	0.52	1.13	2.39	605	1.47					3.71	0.29
"	12	1,900	1.013	5.6	18.5	330	0.53	2.20	3.19	946	1.59	2.84	15.6	12.3	66.3	5.78	0.31

were given.) No salts were added. After the animal had been kept under these conditions for 5 days, daily collections of the complete urinary excretion were made, and the following determinations were made: volume, specific gravity, hydrogen ion concentration by the Clark and Lubs[5] method, total nitrogen by the Kjeldahl method, uric acid by the Benedict-Hitchcock[6] modification of the Folin-Denis method, creatinine by Folin's colorimetric method, creatine by the Folin-Benedict[7] method, ammonia nitrogen by Folin's aeration method, urea by Van Slyke and Cullen's[8] modification of Marshall's urease method, the ammonia formed being titrated, and total phosphates by titration with uranium acetate. The animal was bled four times at intervals

TABLE II.

Period Averages of Urinary Analysis of Pig A.

Date.	Total nitrogen.	Creatinine.	Creatinine N from total N.	Creatine as creatinine.	Creatine N from total N.	Uric acid.	Uric acid N from total N.	Ammonia N.	Ammonia N from total N.	Urea N.	Urea N from total N.	Phosphates as P_2O_5.	$\frac{P_2O_5}{N}$
1919	*gm.*	*gm.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	
Jan 11–17	17.2	1.61	2.52	337	0.61	320	0.56	2.59	15.4	11.9	69.2	3.95	0.23
" 18–24	17.8	1.66	2.51	484	0.84	358	0.60	3.09	17.0	12.0	68.0	4.99	0.28
" 25–31	15.8	1.62	2.77	520	1.03	274	0.52	3.06	19.4	10.1	64.1	5.45	0.34
Feb. 1– 7	20.9	1.90	2.44	823	1.23	340	0.49	4.25	20.3	12.7	60.8	7.07	0.34
" 8–12	18.3	1.73	2.64	832	1.47	334	0.54	2.95	15.2	15.2	67.6	4.88	0.28

of 7 days, in the ordinary manner from the tail, approximately 6 cc. of blood per pound of body weight being taken. The results of the daily analysis of the 24 hour collection of urine are given in Table I, and the period averages of these same data in Table II. The breaks in Table I indicate the occurrence of hemorrhage. The results of the blood analysis are given in Table III. The determinations, in this case, were made as described previously,[1] with the exception that the ammonia in the urea determinations was titrated

[5] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

[6] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.

[7] Benedict, S. R., *J. Biol. Chem.*, 1914, xviii, 191.

[8] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.

instead of Nesslerized, a 5 cc. sample of blood being taken. The alkaline reserve values from this series of bleedings are plotted in Fig. 1. In general, the data illustrate the points which have already been discussed.[1] Of particular interest are the results

TABLE III.

Effect of Hemorrhage on Blood of Pig A.

Date.	Blood taken.	Duration of bleeding.	Time of sampling.	Alkaline reserve.	Total N.	Urea N per 100 cc. of blood.	Remarks.
1919	*cc.*	*min.*		*vol. per cent*	*per cent*	*mg.*	
Jan. 17	1,900	16	Beginning of bleeding.	48.1	2.89	26.3	Fairly quiet throughout.
			End of bleeding.	44.9			
			120 min. after end.	44.9	2.59	32.0	
" 24	2,100	18	Beginning of bleeding.	50.7	2.45	32.4	Fairly quiet throughout.
			End of bleeding.	50.7			
			15 min. after end.	50.7			
			120 min. " "	50.7	2.20	35.6	
" 31	1,850	30	Beginning of bleeding.	51.6	2.55	33.7	Quiet at first. Struggled considerably toward end of bleeding. Had to cut the tail to get the 15 min. sample.
			15 min. after end.	37.4			
			120 " " "	51.4	2.16	38.5	
Feb. 7	1,850	19	Beginning of bleeding.	53.4	2.53	26.0	Quiet at first. Struggled toward end of bleeding. Left pig in crate to take the hour sample but could not get a good stream. Blood clotted very rapidly. Animal quite exhausted but did not develop air hunger.
			15 min. after end.	20.6	2.11	26.5	

obtained at the second bleeding, January 24. In this case, there was no drop in reserve alkali at all, even though a larger amount of blood was taken than at any other bleeding in the series. In the case of the fourth bleeding, February 7, the drop was extremely rapid, but there were no symptoms of air hunger, and the animal apparently suffered no untoward effects from the experiment.

The total nitrogen content was influenced by the hemorrhages much as in the previous experiments. Urea nitrogen was distinctly higher at the end of 2 hours than at the beginning of bleed-

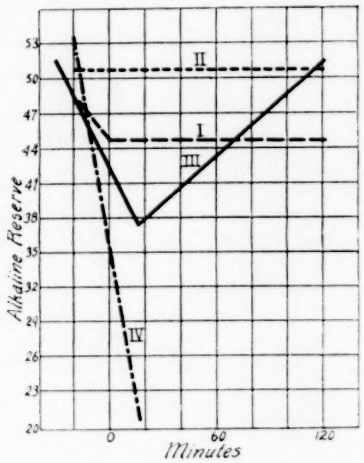

FIG. 1. Curves I, II, III, and IV illustrate the effect of the first, second, third, and fourth hemorrhage on the alkaline reserve of Pig A, which was kept in a metabolism cage and restricted to a diet of corn and distilled water (Tables I, II, and III). All determinations were made by the open vessel method.

ing. In the last bleeding (February 7) a determination of urea nitrogen was made at the end of 15 minutes. At this time the urea nitrogen was the same (a very slight rise) as at the beginning of the bleeding, although the total nitrogen had dropped considerably. In general, bleeding had the effect of raising the urea content of the blood within a short time after the blood was shed, and there was a tendency for the urea to remain high, although it dropped back to the original figure before the end of the experiment, in spite of the fact that the hemorrhages were regularly

repeated at intervals of 7 days. During this time of high urea content in the blood there was no increase in the excretion of urea in the urine (Table II).

In order to study the effect of hemorrhage on endogenous nitrogen metabolism, another animal, Pig B, a female, weighing 360 pounds, was confined in a metabolism cage, and after being fasted for 3 days, was offered distilled water and starch to which sufficient sodium chloride had been added to make the ration palatable enough to induce the animal to eat. After 3 days of starch feeding the sodium chloride was discontinued and daily collections of the urine were made. During the remainder of the experiment the animal received 4 pounds of corn-starch daily and about 5 liters of distilled water. In this way, exogenous nitrogen metabolism was reduced to a minimum, but sufficient calories were provided to cover the energy expenditure of the animal. The results of the daily analysis of the complete urinary excretion are given in Table IV, and the period averages of these results in Table V. This animal was bled twice, and 6 cc. of blood per pound of body weight were taken, the interval between bleedings being 5 days. The alkaline reserve values are given in Table VI.

Soon after the pig was taken from the crate at the end of the 1 hour period, she developed a severe case of air hunger. The respiration was shallow and rapid, about 180 a minute. She refused food and water and was extremely nervous. After several hours an attempt was made to give the animal distilled water by rectum. Several hundred cc. were retained. There was no urine during the first 24 hours after the bleeding. The urine on the following day was collected and analyzed in two separate portions. It was very deeply pigmented. On the following day, March 3, the pig was apparently much better. The respiration seemed normal. She had no difficulty in moving about, but continued to refuse food and water. Consequently the experiment was discontinued, and in an effort to save the animal 3 liters of milk in which 10 gm. of sodium acetate had been dissolved were administered by stomach tube. At night the pig was apparently normal, but was found dead in the morning, March 4, death being caused by a severe hemorrhage of the lungs. There was no reason to believe that death was due directly to the specific effects of the experimental procedure.

TABLE IV.

Daily Analysis of Urine of Pig B.

Date.	Urine.	Reaction.	Specific gravity.	Total N.	Creatinine.	Creatinine N from total N.	Creatine as creatinine	Creatine N from total N.	Uric acid.	Uric acid N from total N.	Phosphates as P_2O_5.	$\frac{P_2O_5}{N}$
1919	*cc.*	*pH*		*gm.*	*gm.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	
Feb. 20	0											
" 21	5,200	6.2	1.008	12.9	3.93	8.19	465	1.12	532	1.23	3.44	0.266
" 22	4,150	6.6	1.006	9.5	1.90	5.46	432	1.41	374	1.17	2.63	0.275
" 23	3,300	6.6	1.005	7.5	2.20	7.88	392	1.63	326	1.30	1.66	0.222
" 24	2,630	6.6		4.6	1.47	8.60	112	0.75	189	1.23	0.92	0.201
" 25	2,000	6.0	1.009	9.0	2.48	7.38	392	1.35	422	1.40	2.12	0.235
" 26	2,900	6.4	1.006	7.6	1.63	5.70	347	1.41	249	0.97	0.95	0.124
" 27	2,725	6.0	1.006	7.3	1.74	6.35	204	0.86	252	1.02	1.80	0.244
" 28	4,300	6.0	1.003	7.8	2.53	8.66	358	1.42	288	1.10	1.83	0.233
Mar. 1	3,700	5.8	1.002	5.0	2.22	11.83	345	2.12	304	1.81	1.13	0.225
" 2	0											
" 3	2,960			27.0	2.93		7,202	8.31	580	0.64	6.60	0.244
" 3*a**	1,460	6.0	1.021	12.2	1.58	3.47	3,789	9.62	292	0.71	3.50	0.28
" 3*b**	1,500	6.2	1.018	14.7	1.34	2.46	3,412	7.22	288	0.59	3.09	0.21

* The urine excreted during this day was collected and analyzed in two separate portions, *a* and *b; a* represents the period from 8 a.m., March 2, to 5 p.m., March 2; *b* represents the period from 5 p.m., March 2, to 8 a.m. March 3.

The breaks in the table indicate the occurrence of hemorrhage.

TABLE V.

Period Averages of Urinary Analysis of Pig B.

Date.	Total N.	Creatinine.	Creatinine N from total N.	Creatine as creatinine.	Creatine N from total N.	Uric acid.	Uric acid N from total N.	Phosphates as P_2O_5.	$\frac{P_2O_5}{N}$
1919	*gm.*	*gm.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*gm.*	
Feb. 20–24	6.9	1.904	7.39	280	1.26	284	1.23	1.73	0.25
" 25–Mar. 1	7.4	2.125	7.70	329	1.33	303	1.18	1.57	0.20
Mar. 2–3	13.5	1.467	2.92	3,600	8.31	290	0.64	3.30	0.24

TABLE VI.

Effect of Hemorrhage on Blood of Pig B.

Date.	Blood taken.	Duration of bleeding.	Time of sampling.	Alkaline reserve.	Total N.	Remarks.
1919	*cc.*	*min.*		*vol. per cent*	*per cent*	
Feb. 24	2,100	30	Beginning of bleeding.	53.8	3.06	Pig was quiet until the last ½ hr. Then she struggled intermittently. Seemed exhausted at the end of the hour. Respiration was heavy and deep, about 70 per min.
			End of bleeding.	56.0		
			15 min. after end.	56.0		
			30 " " "	53.8		
			60 " " "	43.9	2.86	
Mar. 1	2,100	45	Beginning of bleeding.	49.0	2.74	Pig fairly quiet while in the crate. Struggled a little intermittently during the last ½ hr.
			15 min. after end.	36.6		
			30 " " "	44.8		
			60 " " "	30.3	2.45	

DISCUSSION.

The data obtained on the effect of repeated hemorrhage on the composition of the blood of pigs (Tables III and VI, and Figs. 1 and 2) supplement the data already reported in the previous paper. In one case, Fig. 1, Curve II, there was no drop in alkaline reserve after hemorrhage.

The data obtained from the urinary analysis of Pig A (Tables I and II) give no definite indication of increased nitrogen excretion due to hemorrhage. Although the collections were made at the same time each day, it is evident that in some cases the urine so obtained in reality represented a longer period than 24 hours, and in other cases a shorter period. When period averages are considered, the values vary in each direction and therefore cannot be interpreted as demonstrating a significant change. In the case of Pig B, however, where the nitrogen excretion represented endogenous metabolism only, there is a definite indication of increased nitrogen metabolism. This fact might be interpreted

as meaning that when no protein was furnished from which the animal could obtain fragments necessary to replace the lost tissue, there was greater autolysis of body tissue to made good the loss and also greater activity of the blood-making organs with a correspondingly increased excretion of the end-products of the metabolism of these organs. Since the data on the effect of hemorrhage on endogenous nitrogen metabolism is so limited, and since the experiment terminated fatally to the animal, leaving room for doubt as to the exact nature of the cause of death, too much stress should not be placed on the results.

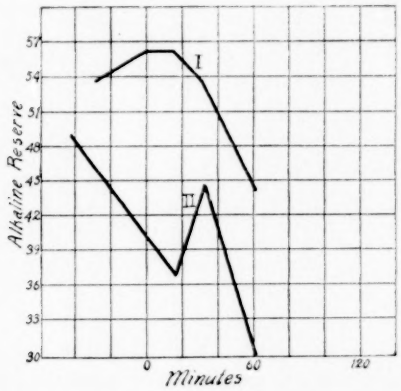

FIG. 2. Curves I and II (open vessel method) illustrate the effect of the first and second hemorrhages on Pig B. The pig was kept in a metabolism cage and the diet was restricted to starch and water. The last point on Curve II represents a sample of blood taken just before severe symptoms of air hunger set in (Tables IV, V, and VI).

In the case of Pig A the effect of hemorrhage on uric acid metabolism was too small to be significant. According to the theory of Lewis and his coworkers,[9] amino-acids stimulate all cellular metabolism, with the result that after protein ingestion (in man) there is an increased excretion of uric-acid. If hemorrhage causes autolysis of tissue protein, an increased excretion of uric acid might be expected. Uric acid, however, is not the only end-product of purine metabolism in the pig. Under

[9] Lewis, H. B., Dunn, M. S., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 9.

the stress produced by hemorrhage, it is conceivable that purine metabolism might be carried out of its normal course. In the case of Pig B, where uric acid excretion was entirely endogenous, the effect of the first bleeding is not clear. If amino-acids stimulate uric acid excretion, it would be expected that total nitrogen and uric acid would run parallel and consequently that the per cent of uric acid nitrogen would not rise. The fact that there was an increased uric acid excretion on the day following bleeding does not necessarily mean that there was an increased production of uric acid during that day, because of the uncertainty of the

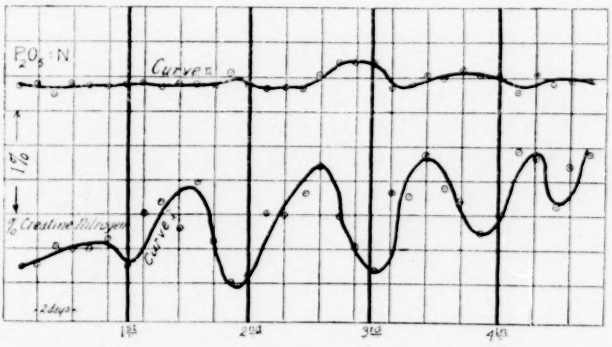

FIG. 3. Curve I represents the daily urinary fluctuation in the per cent of creatine nitrogen of total nitrogen. Days on which bleeding took place are represented by vertical lines. Bleeding stimulates creatine elimination. Curve II represents the daily fluctuation in the ratio of P_2O_5 to nitrogen. This ratio rises after the 18th day, and falls again on the day following bleeding (Pig A, Table I).

accuracy with which the samples represented a 24 hour period. The period averages show a slight increase in uric acid production after the first bleeding, too small, however, to be significant. If the first collections of urine after the second bleeding (there was no urine on the first day) represented a 48 hour excretion, then the actual amounts of uric acid excreted remained remarkably constant. In any case, the uric acid excretion was not at all proportional to the total nitrogen. Although there is no experimental evidence for this view, it is conceivable that those tissues which are comparatively low in nucleic acid, such as the

muscles, were autolyzed to a great extent, with the result that uric acid excretion was not proportional to nitrogen excretion.

Much emphasis has been laid on the constancy of the creatinine excretion for each individual. Haskins,[4] however, found a significant decrease in the creatinine excretion following hemorrhage. It is true that in the case of Pig A there was considerable daily variation in the amounts of creatinine excreted. The percentages of creatinine nitrogen were fairly constant, however, particularly when period averages are considered. It happens that in each case there was a slightly diminished excretion of creatinine nitrogen, expressed as percentage of total nitrogen, on the day following bleeding. These drops fall well within the limits of daily variation, however, and therefore cannot be considered significant. An apparent fall in the per cent of creatinine nitrogen might, of course, be due to a corresponding increase in total nitrogen excretion. This experiment, therefore, affords no evidence that repeated hemorrhages of this magnitude influence the excretion of creatinine. In the case of Pig B, the period averages might seem to indicate that there was a significant reduction in creatinine output after the second bleeding. This effect, also, is directly dependent upon the accuracy with which this collection of urine represented the excretion of the 48 hours following bleeding. Since this point is in doubt, there is little reason to think that the creatinine value was actually lowered by bleeding.

In the case of Pig A, there seems to be no doubt that hemorrhage increased the excretion of creatine when both actual amounts of creatine and percentage of creatine nitrogen are considered. According to the conception of Underhill,[10] that acidosis is a determining factor in the excretion of creatine (in rabbits) this result might be explained on the basis of the fact that hemorrhage is accompanied by lowered alkaline reserve. Several facts, however, are in opposition to this view. The data here presented on the effect of hemorrhage on alkaline reserve, ammonia excretion, and the hydrogen ion concentration of the urine, all point toward the fact that the acidosis produced by this experimental procedure was usually slight and of brief duration. Steenbock and Gross,[11]

[10] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

[11] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxvi, 265.

working with pigs, found that intensive protein-feeding resulted in increased creatine production even during alkalosis. The conclusion seems more plausible, therefore, that the increased creatinuria was caused by an alteration in the course of nitrogen metabolism due to hemorrhage.

When the exogenous nitrogen metabolism was eliminated (Pig B), the second bleeding brought about a tremendous rise in the

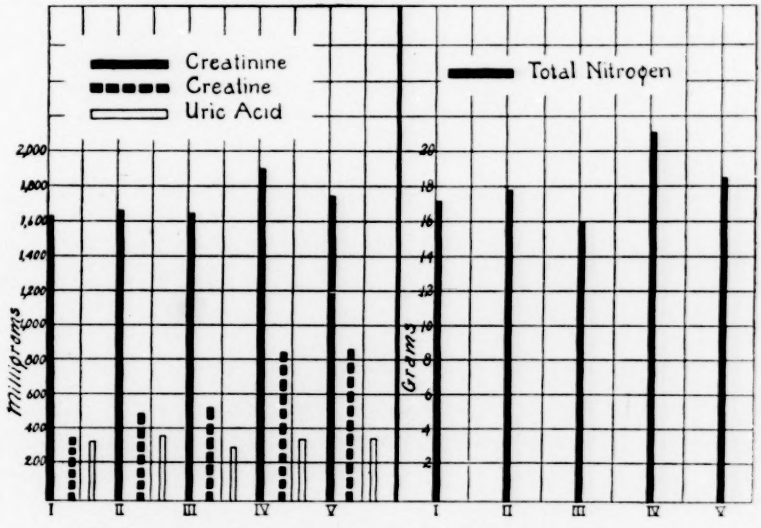

FIG. 4. The vertical lines represent the averages of the actual amounts of creatinine, creatine, uric acid, and total nitrogen respectively excreted during the various periods of the experiment. Line I represents the fore period, Line II the period between the first and second hemorrhages, Line III the period between the second and third hemorrhages, etc. (Pig A, Table II).

amount of creatine excreted, and also in the per cent of creatine nitrogen. It is true that other unknown factors beside simple hemorrhage may have entered into this effect. The picture suggests itself, however, that when the blood stream is suddenly flooded with the fragments resulting from the breaking down of protein of either endogenous or exogenous origin in such large amounts that the organism is unable to metabolize them in the

ordinary way, an increased creatine production and excretion results.

There are considerable data in the literature to show that acid-feeding, if sufficiently intensive, produces an increased excretion of phosphates in the urine in animals. Goto[12] found that acid-feeding produced no definite influence on the distribution of phosphates between urine and feces of rabbits. The data for Pig A show that the phosphate excretion in the urine was fairly constant until the 18th day of the experiment. At this point the ratio $\frac{P_2O_5}{N}$ rose definitely. This fact shows clearly, if it is assumed that there was no corresponding fall in fecal phosphate, that alkaline phosphates were being lost from the body, presumably to help neutralize the excess acid formed. The fact that the next two bleedings were followed on the next day by a decreased $\frac{P_2O_5}{N}$ ratio may indicate that the readily available phosphates were exhausted at the time, due to loss of large quantities of phosphoric acid in the blood withdrawn. In the case of Pig B, the $\frac{P_2O_5}{N}$ ratio remained fairly constant throughout. This effect would be expected on account of the short duration of the experiment; but it is of particular interest inasmuch as the nitrogen figure rose so high after the second bleeding, and no phosphates were supplied with the food.

It is a rather remarkable fact that the hydrogen ion concentration of the urine of Pig B suffered so little change after the second bleeding during the 48 hours when the animal was suffering from acute air hunger.

In the alkaline reserve values (Table VI) obtained after the second hemorrhage is found an interesting example of a preliminary fall in alkaline reserve with the usual attempt at rapid restoration, followed by a second sharp fall. It is unfortunate that samples of blood could not be drawn during the entire period of air hunger.

[12] Goto, K., *J. Biol. Chem.*, 1918, xxxvi, 355.

CONCLUSIONS.

1. On a diet of corn and water, under conditions of repeated hemorrhage, the creatine excretion of two animals (pigs) was definitely increased. This effect was cumulative.

2. When the diet was restricted to starch and water, the second hemorrhage caused an increased excretion of total nitrogen, phosphates, and creatine.

3. The theory that hemorrhages amounting to 6 cc. per pound of body weight are not necessarily accompanied by a severe grade of acidosis is supported by the following observations.

(*a*) The drops in alkaline reserve were slight and of short duration.

(*b*) There was no great increase in the ammonia nitrogen excretion after hemorrhage.

(*c*) The hydrogen ion concentration of the urine was not definitely influenced by hemorrhage.

ANIMAL CALORIMETRY.

Sixteenth Paper.

THE INFLUENCE OF LACTIC ACID UPON METABOLISM.*

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(Received for publication, September 15, 1919.)

The experiments of this laboratory have demonstrated that a causative element of the great increase in heat production after meat ingestion is due to the stimulus provided by the acid products of protein metabolism. Thus, when glycocoll and alanine are given to a dog the metabolism increases greatly (1, 2). The increased metabolism is not due to the process of deamination or of urea formation because the administration of glutamic acid (1) or of asparagine (3) (the latter having the same NH_2 content as glycocoll) is without influence upon metabolism. The increased function of the kidney is without influence, for neither urea nor sodium chloride, when administered in aqueous solution, has any power to increase the basal metabolism (4). These experiments also exclude an increased osmotic exchange between the blood stream and the cells as a cause of the specific dynamic action.

It might be objected that glycocoll and alanine, which sometimes produce vomiting, cause an increased metabolism through gastric or intestinal irritation, but urea and salt solutions also sometimes produce vomiting; yet when they are retained by the stomach there is no rise in heat production after their administration. Benedict and Emmes (5) have shown that the adminis-

* An abstract of this work was published as, Calorimétrie comparée de l'ingestion de viande, d'acide lactique et d'alanine chez l'animal (Lusk, G., *Compt. rend. Acad.*, 1919, clxviii, 1012).

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tration of severe cathartics does not cause the heat production to increase. Furthermore, the increased metabolism is not due to the sudden availability of glycocoll or of alanine as providers of heat because a considerable increase in metabolism is achieved when these substances are administered in phlorhizin glycosuria, under which conditions they are completely converted into glucose and urea without the liberation of energy by oxidation (2). The amino-acids themselves are not stimuli to metabolism, for Rubner (6, *a*) has correctly shown that that quota of the ingested protein which is absorbed as amino-acids and deposited as new protein in the body exercises no *specific dynamic* action, in other words, does not raise the heat production.

There is a fundamental distinction between the heat production induced by protein and that brought about by carbohydrates. Thus, when a fasting dog is caused to perform a measured quantity of work, his heat production is the same as when he is given sugar in abundant supply. If the dog is given meat and caused to do the same amount of work as before his energy production will be raised above the basal level by the increment of the *specific dynamic* influence of the meat superimposed upon the increment of metabolism produced by the performance of the work (7).

In this fact lies the proof that the character of the cause of the increased heat production after giving meat and carbohydrate is essentially different.

That the primary metabolites are different is also indicated by the fact that, though fructose, when given in phlorhizin glycosuria, is converted into glucose and as such completely eliminated in the urine, the intermediary metabolites involved in this chemical transformation do not cause any increase in the metabolism of the dog (2).

In this connection it is of interest to note that the alkaline reserve of the blood falls after the administration of meat, which indicates the production of acid métabolites; this is not in evidence after the ingestion of a vegetarian diet (8). Atkinson and Lusk (3) have demonstrated that 200 cc. of 0.4 per cent of hydrochloric acid will increase the heat production in the dog from 20 to 21.25 calories per hour.

Furthermore, in phosphorus poisoning, a condition in which large quantities of lactic acid are produced, the heat production

is increased (9). It also appears probable that the rise in heat production in severe anemias is due to the pathological production of lactic acid from carbohydrates, which production may be assumed to take place only in the absence of an adequate oxygen supply to the cells (10).

These considerations naturally lead one to the conclusion that lactic acid itself, if given to a dog, might cause an increase in the heat production, just as ingested alanine does, a presumption which proved to be justified.

Methods.

The dog was given the "standard diet" at 5 p.m.; then either (1) the "basal metabolism" was determined by the calorimeter on the following morning, 18 hours or more after food ingestion, or (2) the material to be investigated was given by stomach tube during the morning and the dog was then placed in the calorimeter at an environmental temperature of 25 or 26°C. The results in the two instances could be compared (2).

EXPERIMENTAL.

It was found that when 10 cc. of *d-l*-lactic acid containing 8 gm. of lactic acid in solution were dissolved in 500 cc. of water, the dog invariably violently vomited the material. In one instance when 500 cc. of pure warm water were administered the dog regurgitated a considerable amount of the ingesta as though the mass were too great to be retained. However, when 500 cc. of water containing 2.5 gm. of Liebig's extract of meat were given the fluid was always retained. Whether this difference was due to the saline content or to the flavoring extractives in the Liebig's extract is unknown, but it is certain that beef broth is more readily retained in the stomach than plain water. When 2.5 gm. of Liebig's extract were added to the 1.6 per cent solution of lactic acid described above the fluid was retained in about half the instances. At one time, after quite frequently repeated administrations, the lactic acid solution was persistently vomited whenever given and the completion of the work was delayed until the dog's stomach had recovered from its abnormal irritability. When the lactic acid

was diluted with 400 cc. (instead of 500 cc.) of water the resulting 2 per cent solution was vomited. The solution was always given at a temperature of 38°C.

Series 1.

In this short series of experiments a comparison of the metabolism, after giving 8 gm. of lactic acid, with that found after giving 8 gm. of alanine was made as shown in Table I.

TABLE I.

Dog XVIII, Series 1.

No. of experiment.	Date.	State of nutrition.	Hours of experiment.	R.Q.	Calories per hour.		Increase over indirect basal (17.8 calories).
					Indirect.	Direct.	
	1918						
20	Dec. 26	Basal metabolism.	1	0.84	17.8	15.9	
21	" 27	Lactic acid, 8 gm.; Liebig's extract, 2.5 gm.; water, 500 cc.	2	0.93	20.9	21.0	+3.1
22	" 31	Alanine, 8 gm.; Liebig's extract, 2.5 gm.; water, 150 cc.	2	0.90	19.3	19.2	+1.5

The urinary nitrogen in Experiment 20 was 0.085 gm. per hour; in Experiment 25 it was 0.113 gm. per hour. In Experiment 22 it was assumed that the metabolism of body protein furnished the same 0.113 gm. of nitrogen per hour as in Experiment 25, that 70 per cent of the total amount of *d-l*-alanine was metabolized, and that of this 23 per cent was metabolized in the 2nd, and 21 per cent in the 3rd hour after the ingestion of the material (11).

Since lactic acid and glucose yield almost the same number of calories per gm. and both yield a respiratory quotient of 1.00, the calculation of the heat production by the indirect method after the usual manner is entirely justified.

The experiments (Table I) indicate that 8 gm. of lactic acid administered in 500 cc. of water increase the metabolism from 17.8 to 20.9 calories, an increment of 3.1 calories, whereas 8 gm. of alanine given in 150 cc. of water cause an increase of only 1.5

calories. Unfortunately, no more alanine was available. The discrepancy between the results was due to the difference in the quantity of water ingested, as appears in the second series of experiments.

Series 2.

It was stated by Rubner (6, *b*) and substantiated in this laboratory that the ingestion of a solution of Liebig's extract of meat

TABLE II.

Dog XVIII, Series 2.

No. of experiment.	Date.	State of nutrition.	Hours of experiment.	R.Q.	Calories per hour.		Increase over indirect (17.4 calories).
					Indirect.	Direct.	
	1919						
34	Feb. 24	Meat, 1,080 gm.	2	0.80	34.0	34.1	16.6
37	" 28	Basal metabolism.	2	0.85	17.3	17.0	
39	Mar. 3	" "	3	0.85	17.6	17.2	
40	" 4	Lactic acid, 8 gm.; Liebig's extract, 2.5 gm.; water, 500 cc.	3	0.88	19.4	18.3	2.0
41	" 5	Basal metabolism.	3	0.82	17.5	16.7	
42	" 11	Liebig's extract, 2.5 gm.; water, 500 cc.	3	0.80	18.5	17.6	1.1
43	" 12	Basal metabolism.	2	0.81	17.1	17.0	
44	" 13	Liebig's extract, 2.5 gm.; water, 500 cc.	3	0.78	18.2	17.5	0.8
45	" 14	Liebig's extract, 2.5 gm.; water, 150 cc.	3	0.84	17.0	16.9	
		Average			19.6	19.1	

was without influence upon the heat production. The increase in metabolism after giving meat extract to Dog XVIII, as shown in Table II, seemed at first difficult of interpretation. However, it was demonstrated by Experiment 45 that when the volume of the fluid administered was reduced from 500 cc. to 150 cc. the trustworthiness of the older experiments was confirmed, for there was then no rise in the heat production.

However, whenever the larger volume of fluid was given there was a rapid elimination of it by the kidney, as is shown in Table III.

It appears, therefore, that during the transportation of a large quantity of fluid through the system the heat production rises about 1 calorie per hour above the basal level (in Experiment 42, 1.1 calories, in Experiment 44, 0.8 calorie). A similar phenomenon must also occur in man in the exercise of the habit of excessive beer drinking.

The result of these investigations modifies the interpretation to be placed upon Experiments 21 and 40 in which lactic acid was administered in 500 cc. of water and increases of 3.1 and 2.0 calories per hour were noted, 1 calorie must be deducted in order to give the true increment of the increase due to lactic acid. This would indicate increases of 2.1 and 1.0 calories due to 8 gm. of

TABLE III.

Quantity of Urine Eliminated after the Ingestion of 500 Cc. of Water Containing 2.5 Gm. of Liebig's Extract.

No. of experiment.	Duration of period.		Total volume.	Volume per hour.
	hr.	*min.*	*cc.*	*cc.*
21	3	4	315	104
24	4	15	420	99
26	4	15	420	99
42	3	50	420	110
44	4		375	94
	Average.....			101

lactic acid, which increases are to be compared with an increase of 1.5 calories due to the ingestion of 8 gm. of alanine (the latter having been given in a quantity of water which would not have increased the heat production).

Having determined that 8 gm. of lactic acid and the same amount of alanine produced comparable effects, it remained to investigate the influence of 8 gm. of glucose upon the heat production. The first two materials under favorable conditions may be completely converted into the third. If the reverse reaction takes place, and if 8 gm. of glucose are to any degree convertible into lactic acid or alanine, the metabolism would surely be increased. This problem is answered in the third series of experiments.

Series 3.

A dog received 8 gm. of glucose dissolved in 150 cc. of water and the results given in Table IV were obtained.

It is evident from these results that glucose given in this small amount is without influence upon metabolism. It had been previously known that the administration of 20 gm. of glucose to a dog was almost without influence upon the basal metabolism (1).

TABLE IV.

Dog XVIII, Series 3.

No. of experiment.	Date.	State of nutrition.	Hours of experiment.	R.Q.	Calories per hour.	
					Indirect.	Direct.
	1919					
57	May 9	Basal metabolism.	3	0.87	16.6	16.8
58	" 10	Glucose, 8 gm.; water, 150 cc.	2	0.91	15.8	15.1

CONCLUSIONS.

1. 2.5 gm. of Liebig's extract of beef dissolved in 500 cc. of water facilitate the retention by the stomach of this quantity of fluid by a dog weighing about 11 kilos.

2. It also prevents at times the regurgitation of 500 cc. of a 1.6 per cent solution of lactic acid.

3. The quantity of heat produced by the dog after the ingestion of 2.5 gm. of Liebig's extract of beef dissolved in 150 cc. of water is the same as that of the basal metabolism, but when the mass of fluid given reaches 500 cc. there is an increase of 1 calorie per hour, during which experimental period the quantity of urine eliminated averages about 100 cc.

4. After allowing for this factor, it appears that the increased heat production after giving 8 gm. of *d-l*-lactic acid is 2.1 and 1 calories and is comparable with that obtained after giving 8 gm. of *d-l*-alanine, 1.5 calories.

5. Following the administration of 8 gm. of glucose in 150 cc. of water there is no rise whatever in the basal metabolism. It seems therefore improbable that either lactic acid or alanine is normally produced in appreciable quantities from ingested glucose.

TABLE V.—*Gener*

Date.	Experiment No.	Time.	CO_2	O_2	R.Q.	H_2O	Urine, basal N.	Calories.					Percentage of heat loss by evaporation
								Protein.	Alanine.	Non-protein.	Indirect.	Direct.	
1918			*gm.*	*gm.*		*gm.*	*gm.*						
Dec. 26	20	11.26–12.26	6.10	5.29	0.84	7.04	0.085	2.25		15.52	17.77	15.93	25
" 27	21	11.30–12.30	8.24	6.07	0.99	7.93	(0.113)*	3.00		18.09	21.09	21.37	21
		12.30– 1.30	7.35	6.13	0.87	7.30	(0.113)	3.00		17.77	20.77	20.68	26
" 31	22	11.30–12.30	7.40	5.69	0.95	6.82	(0.113)	3.00	4.47	11.84	19.31	20.25	20
		12.30– 1.30	6.80	5.78	0.85	6.82	(0.113)	3.00	4.08	12.21	19.29	18.11	22
1919													
Feb. 24	34	12.45– 2.45	12.59	7.71	0.83	18.81	1.57	41.62		−7.40	34.22	35.72	53
		1.45– 2.45	11.64	7.71	0.77	18.45	1.57	41.62		−7.95	33.67	33.51	55
" 28	37	11.00–12.00	6.30	5.52	0.83		0.169	4.48		13.88	18.36	17.52	
		12.00– 1.00	5.47	4.85	0.86	6.50	0.169	4.48		11.75	16.23	16.37	23
Mar. 3	39	11.00–12.00	6.39	5.48	0.85	6.88	0.154	4.08		14.27	18.35	17.34	23
		12.00– 1.00	5.97	5.09	0.85	6.13	0.154	4.08		12.96	17.04	15.64	29
		1.00– 2.00	6.05	5.19	0.85	6.28	0.154	4.08		13.28	17.36	18.67	20
" 4	40	11.00–12.00	6.96	5.64	0.90		(0.154)	4.08		15.04	19.12	17.43	
		12.00– 1.00	7.26	5.81	0.91	7.05	(0.154)	4.08		15.74	19.78	17.92	21
		1.00– 2.00	6.75	5.81	0.85	6.46	(0.154)	4.08		15.38	19.46	19.50	20
" 5	41	11.00–12.00	5.89	4.95	0.86		0.145	3.84		12.78	16.62	16.67	
		12.00– 1.00	6.07	5.47	0.81	7.28	0.145	3.84		14.28	18.12	16.46	26
		1.00– 2.00	5.94	5.39	0.80	6.83	0.145	3.84		13.98	17.82	16.99	24
" 11	42	11.00–12.00	6.19	5.52	0.82		(0.145)	3.84		14.49	18.33	16.57	
		12.00– 1.00	5.81	5.46	0.77	6.77	(0.145)	3.84		14.09	17.93	17.32	23
		1.00– 2.00	6.37	5.81	0.80	6.65	(0.145)	3.84		15.38	19.22	18.90	21
" 12	43	11.00–12.00	5.78	5.06	0.83	6.14	(0.145)	3.84		13.00	16.84	17.14	21
		12.00– 1.00	5.77	5.24	0.80	6.16	(0.145)	3.84		13.48	17.32	16.83	22
" 13	44	11.00–12.00	6.11	5.26	0.84			3.84		13.74	17.58	18.43	
		12.00– 1.00	5.83	5.66	0.75	6.60	(0.145)	3.84		14.64	18.48	17.12	21
		1.00– 2.00	5.96	5.71	0.76	6.61	(0.145)	3.84		14.85	18.69	16.98	22

* Nitrogen values in parentheses are assumed from nearby records of the basal value

Summary of Dog XVIII.

Morning weight.	Behavior of dog.	Food.
kg.		
11.35	Quiet.	Basal metabolism.
11.31	Slight movement. Quiet.	8 gm. lactic acid; 2.5 gm. meat extract; 500 cc. water at 10.35 a.m.
11.26	Slight movement between the 2 hours.	8 gm. alanine; 2.5 gm. meat extract; 150 cc. water at 10.30 a.m.
12.07	Several movements. " "	1,080 gm. meat at 9 a.m.
11.95	Slight movement. Quiet.	Basal metabolism.
11.77	Slight movement. Quiet Slight movement.	Basal metabolism.
11.77	Quiet. " "	8 gm. lactic acid; 2.5 gm. meat extract; 500 cc. water at 10.17 a.m.
	" Slight movement. " "	Basal metabolism.
11.55	Quiet. " Slight movement.	2.5 gm. meat extract; 500 cc. water at 10.20 a.m.
11.50	Quiet. "	Basal metabolism.
11.53	" " "	2.5 gm. meat extract; 500 cc. water at 10.15 a.m.

TABLE V—

Date.	Experiment No.	Time.	CO_2	O_2	R.Q.	H_2O	Urine, basal N.	Calories.					Percentage of heat loss by evaporation of water
								Protein.	Alanine.	Non-protein.	Indirect.	Direct.	
1919			*gm.*	*gm.*		*gm.*	*gm.*						
Mar. 14	45	11.30–12.30	5.82	4.83	0.88	5.65	(0.145)	3.84		12.41	16.25	16.37	20
		12.30– 1.30	5.98	5.40	0.81	5.84	(0.145)	3.84		14.04	17.88	16.28	21
		1.30– 2.30	5.93	5.05	0.85	5.34	(0.145)	3.84		13.07	16.91	18.12	17
May 9	57	11.45–12.45	5.81	4.90	0.86		0.150	3.98		12.46	16.44	15.39	
		12.45– 1.45	5.90	4.57	0.94	7.54	0.150	3.98		11.64	15.62	17.00	27
		1.45– 2.45	6.01	5.35	0.82	7.44	0.150	3.98		13.79	17.77	17.98	24
" 10	58	11.00–12.00	5.70	4.58	0.91		0.155	4.11		11.40	15.51	13.76	
		12.00– 1.00	5.95	4.71	0.92	6.92	0.155	4.11		11.90	16.01	6.33	25

The authors wish to acknowledge the assistance of Messrs. J. T. Sheridan and W. M. Stobbs in carrying out these experiments. Mr. Sheridan, a student of brilliant promise, died of the grippe while the experiments were in progress.

Concluded.

Morning weight.	Behavior of dog.	Food.
kg.		
11.55	Quiet.	2.5 gm. meat extract; 150 cc. water at 10.25 a.m.
	"	
	"	
10.80	"	Basal metabolism.
	"	
	Slight movement.	
10.67	Quiet.	8 gm. glucose; 150 cc. water at 10.24 a.m.
	"	

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THE ANTISCORBUTIC VALUE OF THE BANANA.

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The nutritive value of the banana as determined by its adequacy for the growth or maintenance of albino rats has recently been the subject of investigation by Sugiura and Benedict (1). Rats maintained on banana alone failed to increase in size and died after periods of 16 to 80 days without marked loss in weight. It was suggested that the physical properties of the banana might not be favorable for the utilization of the food materials in the digestive tract of the rat. Further experiments in which the banana supplied only a part of the ration indicated that the banana was lacking in adequate amounts of protein and the water-soluble accessory (water-soluble B). For the cure of infantile scurvy the banana has been observed to have little antiscorbutic value (2), being decidedly inferior to the juice of the potato or orange. No studies of the antiscorbutic value of the banana in experimental scorbutus of the guinea pig have been reported.

Three cases of scurvy developed in the group of rats on an exclusive banana diet in the experiments of Sugiura and Benedict (1). No clinical or postmortem observations are recorded, however. The rat is not commonly considered to be susceptible to experimental scurvy, and McCollum and Pitz (3) have shown that normal growth and reproduction are possible in the rat on diets which produce scurvy in the guinea pig. Similar results have been obtained by Cohen and Mendel (4). Harden and Zilva believe that the antiscorbutic factor is necessary for the normal growth of the rat (5) even though those animals do not develop the typical lesions generally associated with the disease. Like conclusions have been reached more recently by Drummond (6). Tozer (7) has also reported that in guinea pigs on

diets deficient in factors other than the antiscorbutic principle, notably the fat-soluble accessory, histological study may reveal a condition closely resembling that present in experimental scurvy. In view of these facts and of the absence of detailed clinical or autopsy data, it may be considered questionable whether Benedict and Sugiura were dealing with typical experimental scurvy in their rats.

The present series of experiments is concerned with the value of the banana as an antiscorbutic in experimental scurvy of the guinea pig.

EXPERIMENTAL.

The experimental animals were young guinea pigs, for the most part about 300 gm. in weight. The greater number of the animals were purchased from a local dealer, who bred small numbers of them, and in whose pens the sanitary conditions were known to be excellent. In later experiments it became necessary to purchase some animals from wholesale dealers. In all cases, however, the animals were kept under careful observation in the laboratory on mixed diets until it was established that they were in good health. The animals were confined in cages about 30 by 18 inches in dimension which allowed a reasonable amount of exercise. These cages were cleaned daily, steamed out with live steam on alternate days, and once a week were washed out with a weak solution of phenol. Food consumption was determined by daily weighing of the uneaten portion. With the exception of a few days at the outset of the experiments, the containers for the drinking water were sterilized daily. The guinea pigs were weighed on alternate days.

The symptoms which were taken as a criterion of the onset of scurvy were those described by Chick, Hume, and Skelton (8), and by Cohen and Mendel (4). In all cases, the clinical data were checked up by postmortem examination, although it was not possible to secure histological examination of the tissues.

Bananas as the Sole Source of Food.—Sugiura and Benedict (1) have shown that the banana is deficient in protein and water-soluble B, and that adequate nutrition of the white rat is impossible on a diet consisting solely of bananas. Inasmuch as the nutritive requirements of the guinea pig have not been worked

out in such detail as have those of the white rat, it was considered desirable to study the influence of the banana diet on the growth of young guinea pigs. Two typical experiments are presented in Chart 1. Guinea Pig K with an average daily consumption of over 65 gm. of banana lost weight rapidly and died on the 26th day. No clinical signs of scurvy were evident and the postmortem examination of the animal revealed a marked condition of inanition, but no lesions of scurvy. Similar results

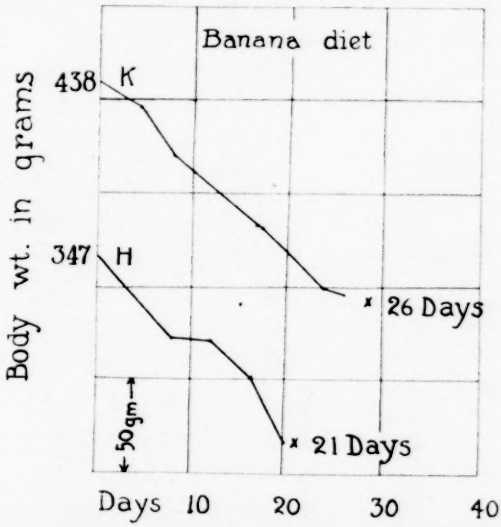

CHART 1. Guinea Pigs H and K received only ripe bananas, the average daily consumption being slightly over 50 and 65 gm. respectively. Both guinea pigs lost weight rapidly and died in 21 and 26 days respectively. No scorbutic symptoms were present at any time.

were obtained with Guinea Pig H whose average daily food consumption was somewhat less. The rapid decline in weight with no accompanying symptoms of scurvy was also shown in experiments (Chart 3) in which after a period of marked loss in weight the banana diet was supplemented by the addition of oatmeal. These results are similar to those reported by Sugiura and Benedict in experiments on white rats, although in the latter the decline in weight was less marked than in the present series.

Lack of the antiscorbutic factor can hardly be included in the list of the deficiencies of the banana as a foodstuff, since despite the marked malnutrition, as evidenced not only by failure of

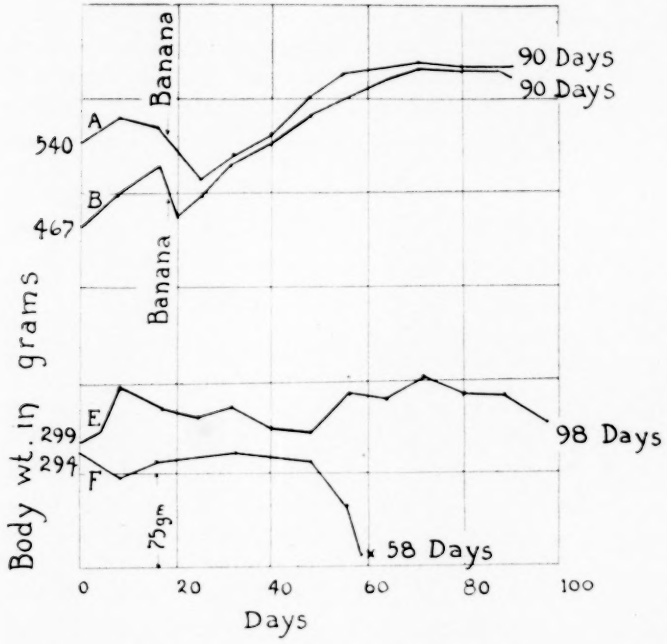

CHART 2. Guinea Pigs A and B received rolled oats *ad libitum* for 18 and 19 days respectively. At this point tenderness of the joints was evident and loss of weight was marked. Banana (35 gm.) added to the diet relieved the scorbutic symptoms and permitted slow growth. The animals were killed after 90 days. No signs of scurvy were observed on autopsy. Guinea Pigs E and F received rolled oats *ad libitum* and 35 and 25 gm. of banana respectively. No scorbutic symptoms developed. The oat intake of Guinea Pig F gradually diminished and the animal died after 58 days. Guinea Pig E was killed at the end of 98 days. Autopsy findings were normal in both cases.

growth, but even of maintenance on this diet, no scorbutic symptoms were observed.

Banana and Rolled Oats.—The early experiments of Holst and Frölich (9) and later investigations from other laboratories (3, 4,

10, and 11) have shown that guinea pigs on an exclusive cereal diet usually die in from 20 to 30 days with scorbutic symptoms. In Chart 2 and Table I are presented typical protocols of experiments in which banana supplemented the oatmeal diet. In the earlier

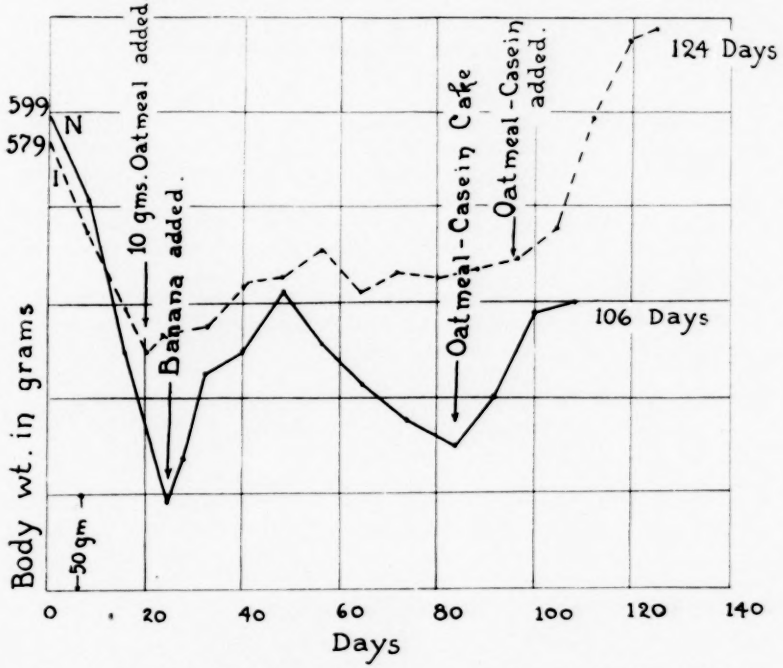

Chart 3. Guinea Pig I received an exclusive banana diet up to the 20th day; 10 gm. of oatmeal were then added until the 93rd day, after which the oatmeal-casein cake was fed in place of the oatmeal. The animal was free from clinical symptoms of scorbutus throughout the period of the experiment. Guinea Pig N received rolled oats only for 24 days. Soreness and slight enlargement of the joints developed about the 18th day. 30 gm. of banana as a supplement to the diet relieved the scorbutic symptoms although satisfactory growth was not made. When the oatmeal-casein cake was substituted for the rolled oats, rapid growth ensued.

part of the experiments bran was also added to the diet, but since the animals chose the rolled oats and left the bran unconsumed its use was discontinued. Provided the daily intake of banana could be maintained at 25 gm. or more, no scorbutic

TABLE I.

Guinea pig No.	Duration.	Weight			Diet.	Notes.
		Initial.	Maximum.	Final.		
	days	*gm.*	*gm.*	*gm.*		
A	90	540	615	608	Oatmeal to 18th day; oatmeal and 35 gm. banana thereafter.	Marked loss in weight 12th to 18th day; slight gain to 60th day; no gain thereafter. Killed. No scurvy.
B	90	467	604	592	Oatmeal to 19th day; oatmeal and 35 gm. banana thereafter.	Marked decline in weight 14th to 20th day; gradual gain to 56th day. Killed. No scurvy.
C	26	687	693	483	Oatmeal.	Marked loss in weight after 18th day. Died. Scurvy.
D	34	320	331	238	Oatmeal and 10 to 15 gm. banana daily; banana eaten in small amounts only after 24th day.	Marked loss in weight after 20th day. Died. Severe scurvy.
E	98	295	358	317	Oatmeal and 35 gm. banana.	No growth; fluctuations in weight. Ate little food after 90th day. Killed. No scurvy.
F	58	290	299	213	Oatmeal and 25 gm. banana; diminished intake of oats toward end of experiment.	Progressive loss in weight with diminished food intake. Died. Inanition. No scurvy.
G	44	329	329	255	Oatmeal and 20 gm. banana.	Prolapse of rectum. Killed. Marked impaction of cecum. Mild scurvy.
H	21	347	364	247	Banana about 50 gm. daily.	Died. Inanition. No scurvy.
K	26	438	438	326	Banana about 65 gm. daily.	" " " "
I	124	579	641	641	Banana alone to 20th day; oatmeal and banana to 93rd day; oatmeal-casein cake thereafter.	Marked loss in weight on banana diet; maintenance on banana-oat diet; vigorous growth on banana and oat cake.
M	73	754	754	416	Banana alone to 20th day; oatmeal and banana to end of experiment: little banana eaten last 2 weeks of experiment.	Died. Marked impaction of cecum. Severe scurvy.

TABLE I—*Continued*.

Guinea pig No.	Duration	Weight.			Diet.	Notes.
		Initial	Maximum	Final.		
	days	*gm.*	*gm.*	*gm.*		
N	106	599	643	505	Oatmeal to 24th day; oatmeal and 30 gm. banana to 75th day; oat cake and 30 gm. banana thereafter.	Loss in weight on oat diet; slight soreness of joints on 18th day; maintenance and slight growth on oat-banana diet; good growth on banana and oat cake. Killed. No scurvy.
S	86	235	431	423	Oatmeal and 25 gm. banana to 30th day; oat cake and 20 to 25 gm. banana thereafter.	Maintenance and slow growth on oat-banana diet; better growth on oat cake and banana. Killed. No scurvy.
T	48	308	308	187	Oatmeal to 12th day; oatmeal and 25 gm. banana to 30th day; oat cake thereafter.	Died. Marked clinical and postmortem symptoms of scurvy.
U	48	270	323	198	Oatmeal to 18th day; oatmeal and banana to 30th day; oat cake and banana to 36th day; oat cake alone thereafter.	Died. Severe scurvy.
V	86	272	491	491	Oatmeal to 18th day; oatmeal and banana to 29th; oat cake and 15 gm. banana thereafter.	Rapid gain in weight after addition of oat cake and banana. No scurvy.
W	86	276	477	477	Oatmeal to 20th day; oatmeal and banana to 28th day; oat cake and 25 gm. banana thereafter.	Loss in weight on oats alone; slight gain on banana-oat diet; rapid gain on oat cake and banana.
Y	24	352	352	223	Oat cake.	Rapid decline in weight after 16th day. Died. Scurvy.
Z	23	517	544	315	" "	Marked loss in weight after 14th day. Died. Mild scurvy.
10	81	230	500	495	Oat cake and 10 gm. banana.	Steady growth. In excellent condition at end of experiment.

TABLE I—*Concluded.*

Guinea pig No.	Duration.	Weight.			Diet.	Notes.
		Initial.	Maximum.	Final.		
	days	*gm.*	*gm.*	*gm.*		
11	83	238	335	332	Oat cake to 23rd day; oat cake and slightly less than 10 gm. banana thereafter; in last 10 days banana intake slightly above 10 gm.	Decline from 13th to 23rd day; increase to 39th day, decline to 49th day; steady gain thereafter. Soreness of joints from 30th to 67th day. In excellent condition at end of experiment.
12	72	258	433	433	Oat çake and 15 gm. banana.	Fair growth, no scurvy.
13	72	338	476	476	Oat cake and 15 gm. banana.	Fair growth; marked increase in weight during last 12 days. No scurvy.
14	72	273	518	518	Oat cake and 20 gm. banana.	General but somewhat irregular growth to 42nd day. Regular growth thereafter.
16	72	443	594	594	Oat cake and 20 gm. banana.	Fair growth. No scurvy.

symptoms were observed. Ingestion of quantities of banana less than this failed to give satisfactory protection against scurvy (Table I, Guinea Pigs D, G). Adult animals (Chart 2, Guinea Pigs A, B) maintained their weight or grew slowly, while younger animals (Chart 2, Guinea Pigs E, F) were barely able to maintain themselves, and in some cases showed marked decline in body weight, but no scorbutic symptoms, either clinical or postmortem. These results would seem to indicate that the banana is greatly inferior to most other fruits and vegetables as an antiscorbutic.

Inasmuch as the oat has been shown (3) to be deficient in the protein, inorganic salt, and fat-soluble A factors, and as the banana is also deficient in at least one of these same factors, it was felt that although the experiments had demonstrated a definite antiscorbutic value of the banana, the results could hardly be judged on the quantitative basis in view of the above mentioned deficiencies of the diet. A greater amount of the anti-

scorbutic principle may be requisite when the diet is not otherwise adequate to provide for normal growth and maintenance.

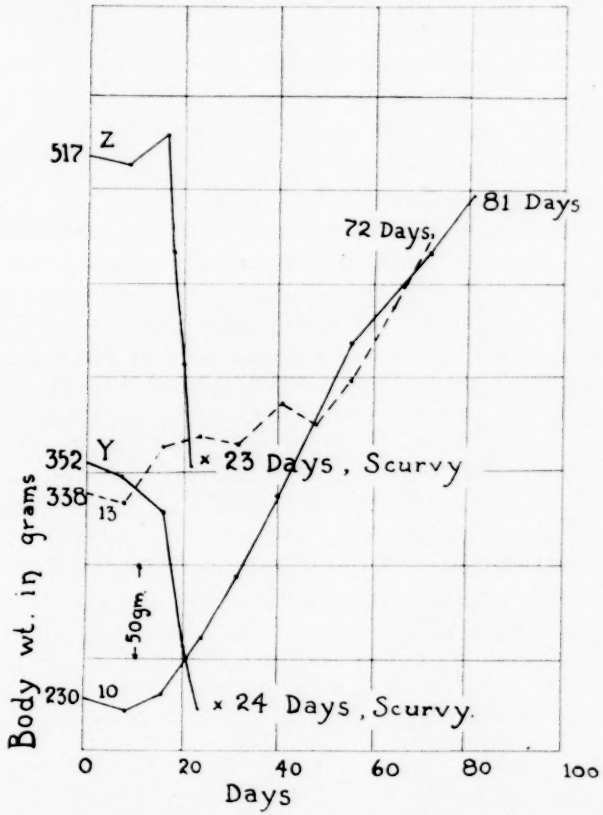

CHART 4. Guinea Pigs Y and Z were maintained on the oatmeal-casein cake without the addition of banana, and developed scurvy which resulted in death. Guinea Pigs 10 and 13 received the same diet with a supplement of 10 and 15 gm. of banana daily. The animals were in excellent condition throughout the course of the experiment.

Banana and Oatmeal-Casein Diet.—For the purpose of rendering the basal diet more nearly adequate for normal growth, the following ration was prepared.

Autoclaved rolled oats	500 gm.
Wheat bran	100 "
Purified casein	40 "
Raw milk	200 cc.
Calcium lactate	15 gm.
Sodium chloride	15 "
Distilled water	400 cc.

The oats were heated in an autoclave for 30 minutes at 120°. The dry ingredients were thoroughly mixed, the liquids added, the pasty mass was spread out in thin sheets, and dried at 75–80°. The resulting cake was relished by the animals so that little difficulty was experienced in securing a daily intake of 20 gm.

Guinea pigs maintained on this oatmeal cake developed scurvy as rapidly as on an exclusive oatmeal diet (Chart 4, also Guinea Pigs T, U, Table I). When this cake was supplemented by banana, not only was scurvy prevented, but rapid growth in young animals also occurred, growth which was in striking contrast to the results observed when banana supplemented an exclusive oat diet. This contrast is clearly shown in Chart 3. With this more complete diet protection against scurvy and normal growth were obtained with 10 to 15 gm. of banana. Thus Guinea Pig 10 (Chart 4) on a diet of 20 gm. of oat cake and 10 gm. of banana daily gained 265 gm. in 81 days, increasing in weight from 230 to 495 gm. Similar results were obtained with the other animals of this group (Guinea Pigs 10 to 16, Table I). It is evident that when supplementing a diet otherwise adequate, the banana has considerable value as an antiscorbutic, although its potency in this respect is not so great as that of the orange or potato.

SUMMARY.

1. Guinea pigs fed on an exclusive diet of bananas are unable to maintain their body weight and die in 20 to 30 days. Autopsy reveals a condition of marked inanition, but no lesions characteristic of scurvy.

2. Bananas in amounts greater than 25 gm. daily as a supplement to a diet of rolled oats prevent the onset of scurvy. Such a diet, however, does not permit normal growth in young animals. Less than 25 gm. of banana as a supplement to the oat diet does not protect against scurvy.

3. Scurvy can be readily produced experimentally on a diet of autoclaved rolled oats supplemented by bran, milk, casein, and inorganic salts. When such a diet is further supplemented by banana, 10 to 15 gm. will serve to protect against scurvy. Such a diet not only affords protection against scurvy but results in rapid growth of young guinea pigs.

4. These experiments suggest that a lower content of the anti-scorbutic principle may be sufficient to protect against scurvy if the diet is adequate in its content of the other essential dietary constituents.

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THE DETERMINATION OF CARBON MONOXIDE IN BLOOD.

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(Received for publication, September 15, 1919.)

The determination of carbon monoxide in blood may be performed by gasometric, colorimetric, or spectrophotometric methods.

Previous gasometric methods have been laborious and most of them required special apparatus (as those of Gréhant, de Saint Martin, Nicloux, and others). The method employed by Zuntz and Plesch (1) seems to be the easiest. They determine the carbon monoxide in 1 cc. of blood, using ferricyanide to set the gases free (CO and O_2) and burn the CO to CO_2, which is absorbed by KOH. They calculate the amount of CO by the difference in pressure as in Haldane and Barcroft's method for blood gas analysis. According to the authors, the expulsion of the blood gases is finished after 1 hour.

The colorimetric method of Haldane (2) is based upon the fact that dilute carmine solutions have nearly the same color as dilute solutions of carbon monoxide hemoglobin. If it is known how much carmine has to be added to normal blood in order to give it the same color as blood completely saturated with carbon monoxide, and it is determined how much of the carmine must be added to normal blood to give it the color of the blood with the unknown content of CO, a simple calculation by proportion will give the degree of saturation with carbon monoxide.

This method seems to be very accurate in Haldane's hands, while others, like Krogh (3), and Zuntz and Plesch (1), have been unable to obtain good results with it. Plesch (4) has modified it, using a hemoglobin solution saturated with carbon monoxide instead of carmine for the titration.

Spectroscopic methods have been used by Hüfner and later by Hartridge (5). Krogh (6) has recently modified the method of the latter and used it for the determination of the oxygen capacity of very small amounts of blood; his method is still rather rough, but he hopes to make it more accurate with finer instruments.

An easy gasometric method seems still to be needed and we have worked out one which permits the determination of the carbon monoxide gasometrically in 2 cc. of blood in the course of 10 to 15 minutes. We have employed this method for the determination of the blood volume and for the study of the action of carbon monoxide on blood.

The Method.

The principle of our method is to set free the oxygen and carbon monoxide from their combination with hemoglobin in the blood by addition of ferricyanide and then to remove both gases with the help of a Torricellian vacuum in the Van Slyke apparatus for blood gas analysis. The oxygen is absorbed in the apparatus by alkaline pyrogallate and the volume of residual carbon monoxide is measured directly at atmospheric pressure, a correction being made for the small and constant amount of nitrogen gas physically dissolved by blood.

The procedure is, up to the time when the expelled gas is measured, exactly the same as that for the oxygen method described by Van Slyke (7), and it is therefore unnecessary to repeat it here; the same amount of blood and the same solutions are used, and only the shaking has to be continued a little longer before a constant reading is obtained. This takes about 2 to 3 minutes and is a little different for different species of blood; it probably depends upon the facility with which the blood is laked.

When the reading of the volume of the gas mixture, consisting of oxygen, carbon monoxide, and a little nitrogen, is constant, a solution of alkaline pyrogallate[1] is introduced into the cup of the apparatus, is covered by a thin layer of paraffin oil, and is allowed to flow slowly down the inner wall of the graduated part of the

[1] Prepared by dissolving 10 gm. of pyrogallic acid in 200 cc. of strong potassium hydroxide (160 gm. of KOH dissolved in 130 cc. of water).

apparatus. A little suction is produced during this part of the procedure by lowering the leveling bulb slightly.

The absorption of the oxygen is very rapid and is completed in less than 1 minute; the reading is taken and the pyrogallate solution introduced once more until a constant reading is obtained. The gas is then measured under barometric pressure in the same way as described by Van Slyke for carbon dioxide (8) and oxygen.

As the solution is very dark and it is a little difficult to get good readings of the meniscus, we have produced a new meniscus by letting a little water flow down after the pyrogallate solution; the water floats on the top of the fluid and one can get readings to about 0.002 cc. Instead of water a few drops of octyl alcohol may be used.

The apparatus is washed out twice with dilute ammonia solution after each determination.

Calculation.—The gas measured is reduced to standard conditions by multiplying by the factor $(0.999–0.0046\ t) \times \frac{\text{Barometer}}{760}$, t being the temperature in °C. If 2 cc. of blood have been used, the values of this factor in Column 3 of Table I of Van Slyke's paper on oxygen[2] may be used, the result then being expressed in cc. of CO per 100 cc. of blood, when the nitrogen correction, 1.2 cc., is subtracted.[3]

EXPERIMENTAL.

Air was analyzed in the Van Slyke apparatus in order to find the best way of absorbing the oxygen. When the pyrogallate solution was introduced in the manner described above, the oxygen was absorbed in 30 seconds.

The method was tried in the following way. As the oxygen and the carbon monoxide replace each other in the combination with hemoglobin and the oxygen capacity equals the carbon monoxide capacity, blood with known percentages of carbon monoxide may be obtained by mixing different amounts of blood

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 130.

[3] The nitrogen correction is 1.2 per cent, instead of the calculated value 0.9 per cent, when actually determined by Bohr and by ourselves.

saturated with carbon monoxide with blood saturated with air. By taking, for instance, one part of CO blood and four parts of O_2 blood, the analysis should give 20 per cent CO and 80 per cent O_2.

Table I shows the amounts of oxygen and carbon monoxide found by analysis and the amount calculated.

TABLE I.

No.	Blood used.	Found.			Calculated.			Kind of blood.
		Oxygen.	CO	Proportion of hemoglobin saturated with CO.	Oxygen.	CO	Proportion of hemoglobin saturated with CO.	
	cc.	*cc.*	*cc.*	*per cent*	*cc.*	*cc.*	*per cent*	
1	2	0.212	0.206	50.7	0.209	0.209	50.0	Guinea pig.
2	2	0.208	0.210	49.8	0.209	0.209	50.0	Same blood.
3	2	0.118	0.240	32.9	0.117	0.238	33.3	Rabbit.
4	2	0.170	0.328	34.1	0.116	0.332	33.3	Ox.
5	2	0.088	0.250	26.0	0.084	0.252	25.0	Rabbit.
6	"	0.128	0.390	24.5	0.129	0.388	25.0	Ox.
7	2	0.068	0.240	22.1	0.062	0.246	20.0	Rabbit.
8	2	0.054	0.224	19.43	0.046	0.231	16.7	"
9	4	0.091	0.434	17.3	0.088	0.437	16.7	Same blood.

It is seen from Table I how closely the found values agree with those calculated, except in No. 8, where the discrepancy is 2.7 volumes per cent. By taking 4 cc. of the same blood instead of 2 cc., this error is brought down to 0.6 volume per cent. We therefore recommend the use of a little more blood, 3 or 4 cc. (and the correspondingly increased amount of ammonia), for analysis, if the percentage saturation of CO is very small and the actual amount of CO found in 2 cc. of blood is 0.05 cc. or less.

In two rather rough experiments, two guinea pigs were given illuminating gas under a bell jar until they fell unconscious. They were then taken out and blood was drawn by heart puncture for analysis. One of them died while the blood was being taken; the other recovered and behaved normally an hour afterwards.

Table II shows that a guinea pig can recover after carbon monoxide poisoning, even when the blood is 76.3 per cent saturated with carbon monoxide.

TABLE II.

	O_2	CO	Saturation with CO.	Remarks.
	cc.	*cc.*	*per cent*	
Guinea Pig 1	0.115	0.353	75.4	Died during bleeding.
" " 2	0.080	0.258	76.3	Recovered.

SUMMARY.

A method is described for the determination of carbon monoxide in blood, the technique of which is exactly the same as that previously described by Van Slyke for the determination of oxygen, except that after the gases are extracted the oxygen is absorbed in the apparatus by introducing alkaline pyrogallate solution. The carbon monoxide remains and is measured directly at atmospheric pressure.

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THE DETERMINATION OF BLOOD VOLUME BY THE CARBON MONOXIDE METHOD.

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There is still much confusion regarding the exact value of the blood volume in human beings, though much work has been done in this field of investigation. Values for the blood volume for men are given, ranging from $\frac{1}{21}$ to $\frac{1}{8}$ of the body weight. These widely differing results have been obtained by the use of different methods.

There are one direct and several indirect methods for the determination of the blood volume.

Direct method.—Welcker (1) in 1854 was the first one to determine the blood volume by a method which still is regarded as the standard; he bled animals to death, washed out the vessels with water, and extracted the hemoglobin still remaining in the tissues by mincing the organs minus the bile and the content of the bowels, and placing them in water for several days. By comparing the hemoglobin content of the first blood and the blood washings and extracts brought together, he found the blood volumes of mammals to constitute $\frac{1}{13}$ of the body weight. The same value was obtained for human beings by Bischoff (2), who used this method on two criminals.

Welcker's method has been modified and improved by several investigators, but the general principle is the same as in 1854. The results obtained in animals with this method have differed because of incomplete washing and extraction, and the use of inexact methods for the hemoglobin determinations.

Indirect methods.—These methods must be used in experiments on living animals. There are various principles for the indirect determination of the blood volume. The best of them may be divided into two groups: (1) A known amount of an easily determinable substance, which is kept within the circulatory system for a sufficiently long time for thorough mixing, is introduced into the blood and the concentration of it deter-

mined. (2) The blood is either (*a*) diluted, or (*b*) concentrated in various ways, and the blood volume calculated from the variation in the content of hemoglobin or corpuscles.

Group 1. (*a*) *Carbon Monoxide Method.*—The principle of this method is to administer a certain amount of carbon monoxide gas to the individual, and then to determine the degree of saturation of the blood with CO or the actual amount of CO per unit of blood. It was first used by Gréhant and Quinquaud (3), and later by Haldane and Smith (4), Oerum (5), Douglas (6), Boycott and Douglas (7), and Plesch (8). (*b*) The dye method of Keith, Rowntree, and Geraghty (9) is the method most widely used for the present in clinical work. A dyestuff, vital red, is injected intravenously and determined colorimetrically in the blood. The relative amounts of plasma and corpuscles are determined by the hematocrit method. (*c*) The antitoxin method of von Behring (10) is based upon the observation that tetanus antitoxin remains in the circulation for a long period; a known amount is injected, and the concentration then determined in the blood. (*d*) The acacia method of Meek and Gasser (11) has been tried only in animals so far, and the experience is not large enough to judge of its utility. Acacia is injected and determined in the blood as furfurolphloroglucine.

Group 2. (*a*) *Dilution Methods.*—The only one of these methods which has withstood criticism is based upon the observation of Cohnstein and Zuntz (12) that isotonic sodium chloride solutions are kept in the circulation for a relatively long time, and diffuse very slowly out into the tissues; this is utilized for the determination of the blood volume, the red cells being counted before and after infusion. Plesch (8) seems to have developed this method to further exactness by determining the hemoglobin instead of the cells with the help of his chromophotometer. As the variations in the cell or hemoglobin content obtained by dilution hardly exceed 10 per cent, the exactness of the method evidently depends on how accurately these constituents of the blood can be determined.

(*b*) *Concentration Methods.*—The method of Tarchanoff (13), who determined the hemoglobin before and after a steam bath and the decrease in weight through loss of water, and calculated the blood volume from these two factors, has been justly criticized and cannot be relied upon, as the water may be derived from other sources in the body than the blood.

Quincke (14) transfused blood with a certain amount of red corpuscles to two anemic patients and calculated the blood volume from the increase in the red count. Lindeman (15) uses the same principle. These methods can only be used in anemia.

Of these methods only three are of practical value for physiological and clinical purposes; they are the carbon monoxide method, the infusion method of Cohnstein and Zuntz, and the vital red method.

The results obtained by these methods vary. In animals, both the carbon monoxide method and the infusion method have given nearly the same values as the Welcker method; Gréhant and Quinquaud (3) found in nine dogs values from $\frac{1}{11}$ to $\frac{1}{13.8}$ of the body weight with the CO method,

which correspond to Welcker's own results in dogs. Douglas (6) found a close agreement between the CO method and the bleeding method in five rabbits; Boycott and Douglas (7), repeating the experiments later, found a little higher value with the CO method (2 per cent). Plesch (8), in dogs, tried subsequently the CO, the infusion, and the bleeding method, and the results were uniform. The vital red method has never been checked up by the Welcker method as far as can be seen from the literature.

In human beings the results are widely different. Bischoff's values, $\frac{1}{13}$ of the body weight, were regarded as the standard until Haldane and Smith (4), with the CO method and carmine titration, in fourteen normal men found $\frac{1}{21}$, the highest value being $\frac{1}{16}$ and the lowest $\frac{1}{30}$ (in a very fat man). Oerum (5), using the same technique, found in men $\frac{1}{19.2}$, and in women $\frac{1}{21.8}$. Plesch (8), using a gasometric method for the CO determination in four men, found the average ratio $\frac{1}{17.9}$; also in five persons, some of whom were reported to be fat, he determined the blood volume with the infusion method and found $\frac{1}{19.1}$. Bischoff's results, therefore, seem to be too high, inasmuch as the two criminals were hardly normal individuals, one at least suffering from scurvy. The method used is also open to criticism.

Douglas (16), in 1910, made a series of determinations with the CO method on himself and another subject with Haldane's technique, but waited a longer time before he took the blood sample for analysis. These results show a mean value of $\frac{1}{13.9}$ for his own and $\frac{1}{12.5}$ for the other subject, values more in accordance with those of Bischoff. He found errors in Haldane and Smith's determinations due to incomplete mixing of the blood, as the blood sample was taken too early after the breathing of the carbon monoxide.

In 1915 Keith, Rowntree, and Geraghty (9), with their vital red method, found still higher values, the mean in normal men being $\frac{1}{11.8}$ of the weight. This method has never been controlled by the Welcker method as far as can be seen, but the authors show its reliability toward relative changes by drawing a certain amount of blood and finding a corresponding drop in the blood volume.

It may be seen from this review of the literature how uncertain is our knowledge of the blood volume in human beings. Since the carbon monoxide method in animals has given satisfactory results as compared with the standard method of Welcker, and

in human beings the results obtained by the various investigators have differed widely, it seems worth while to make further investigations in this field, especially since the technique used before has been rather difficult.

It seems certain, according to Douglas, that Haldane and Smith's figures are too small, and so must be the figures of Oerum, as he used the same technique. There remain, therefore, only the determinations of Plesch in four persons, giving the average of $\frac{1}{17.9}$ and those of Douglas, on himself and another man, giving the values of $\frac{1}{13.9}$ and $\frac{1}{12.5}$ of the body weight.

The carbon monoxide method has been criticized by Dreyer (17) and his coworkers, who, in rabbits, used Haldane and Smith's technique, and got so much divergence in the figures that they concluded it could not be used in its present form. They therefore determined the blood volume by injecting in rabbits' blood a known amount of agglutinin, determined the percentage in the serum, then washed out the circulation, and determined the percentage of agglutinin in the washing. They claim the blood volume to be a function of the surface area, so that, for instance, smaller rabbits have a relatively higher blood volume than the larger ones.

The adverse criticism of the carbon monoxide method may be due to the difficulty of the technique, as the carmine titration of Haldane requires long training and a highly developed color sense. All the carbon monoxide determinations in the present paper are performed with the help of the gasometric method, described in the preceding paper, which makes the whole technique much simpler and fitted for general use.

Bood Volume Determinations in Animals.

The results of numerous determinations of Boycott and Douglas (7) show that rabbits' blood constitutes from $\frac{1}{22}$ to $\frac{1}{18.1}$ of the body weight, as determined both with the carbon monoxide and the washing out method. The average of 52 rabbits with the washing out method was $\frac{1}{20.9}$, or 4.77 cc. of blood per 100

gm. of body weight, while the carbon monoxide method (with the carmine titration) gave a little higher result, $\frac{1}{18.1}$ or 5.5 cc. per 100 gm.

Methods.

The arrangement used was that described by Douglas (6). It is therefore unnecessary to repeat it here.

The principle is to let the rabbit breathe into a closed system which is supplied with arrangements for removing the carbonic acid and renewing the oxygen. A measured amount of carbon monoxide is introduced into the apparatus, and 10 minutes after the entire amount is given a sample of blood is drawn from the ear vein and analyzed for carbon monoxide. At the same time a sample of the air in the chamber is taken for determination of the O_2, CO_2, and CO. The blood volume is calculated from the amount of carbon monoxide absorbed by the animal and the concentration of it in the blood. As in Douglas' experiments, a tube connected with a bell jar, partly immersed in a glass of water, was introduced into the respiratory chamber, and served as an indicator of the pressure in the apparatus. While the air sample was drawn, the oxygen current was cut off and the water allowed to rise in the bell jar by raising the glass in order to compensate for the negative pressure produced by the sucking out of the air.

The capacity of the apparatus (the chamber and the air in the rubber tubing, the pump, and the bell jar) was 750 cc., a little larger than that of the apparatus employed by Douglas (6), and Boycott and Douglas (7).

The carbon monoxide was prepared by heating oxalic acid and concentrated sulfuric acid, and the gas was collected over water made alkaline with sodium hydroxide, with which the gas was shaken in order to get rid of the CO_2. The gas was analyzed every 2nd day by shaking it in a Hempel pipette with cuprous chloride solution, which absorbs the carbon monoxide and O_2, and from the amount of nitrogen left the air content of the gas was calculated. The gas first evolved was discarded. The carbon monoxide content of the rest was 95 to 98 per cent.

The carbon monoxide in the blood was determined by the method described in the preceding paper, the blood, 4.5 cc. in

all, being drawn from the ear vein without stasis and kept under paraffin oil; 2 cc. were used for each analysis. The hemoglobin was determined by the Palmer method (18). The air in the chamber was analyzed for O_2 and CO_2 in the Haldane-Henderson apparatus.

The determination of the carbon monoxide left in the chamber could not be made by gasometric methods, and the method of Haldane (19) was employed, the principle of which is to shake the carbon monoxide-containing air with blood, to estimate colorimetrically the percentage of saturation of the blood with carbon monoxide, and deduce from this value, and the percentage of O_2 present, the percentage of carbon monoxide in the air. As bloods of different species and also of different individuals show different dissociation curves for the carbon monoxide hemoglobin, as shown by Krogh (20) and Haldane (21), blood from a single sheep was used, in which the dissociation curve was previously determined (preceding paper). The dissociation curve is a hyperbola of the formula

$$\frac{(O_2 \text{ percentage in air})}{(CO \text{ percentage in air})} \times \frac{Hb\,CO}{Hb\,O_2} = K$$

In the blood used $K = 179$ at 24°.

For the determination of the dissociation curve of the blood of our sheep, 5 cc. samples of the blood were rotated in bottles of known capacity (approximately 1 liter), filled with air plus known amounts of carbon monoxide. The blood was first placed in the bottle, which was then closed by a stopper containing a three-way capillary cock. A known volume (1 to 4 cc.) of analyzed CO gas was then forced in from a micro-gas-burette, in which the volume delivered could be read over mercury to within 0.002 cc. The tubes of the cock were filled with the CO before the measured amount was admitted into the bottle, so that errors due to dead space were avoided. The bottle with the blood and gas mixture was rotated for 2 hours at 24°C. Trial showed that equilibrium was obtained in this time. Samples of 2 cc. of blood were then withdrawn and used for the determination of O_2 and CO as described in the foregoing paper. The volume of CO taken up by the 5 cc. of blood was subtracted from the volume of CO originally added, in order to estimate the amount left in the gas

phase. The oxygen concentration remained that of atmospheric air.

The results obtained in four determinations are shown in the curve of Fig. 1. The curve is the hyperbola plotted from the formula, with 179 taken as the value of K; the crosses represent the results experimentally obtained.

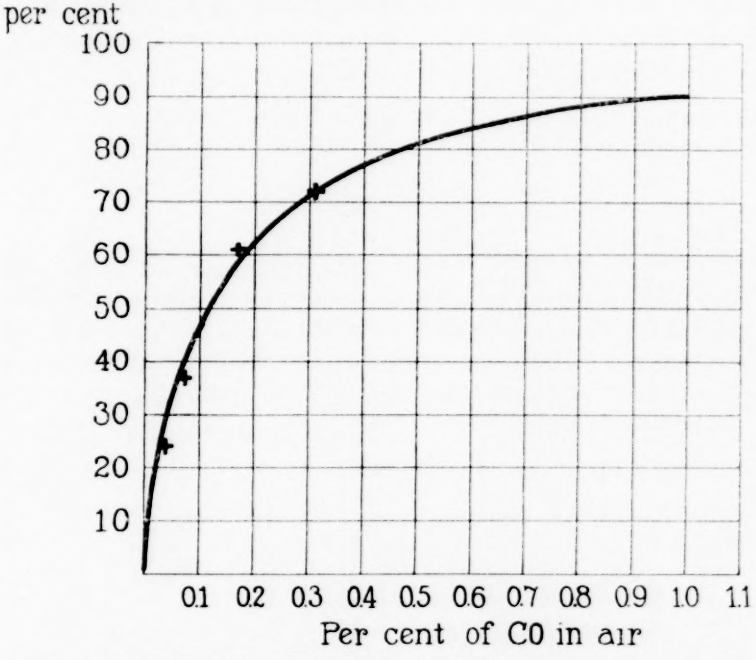

FIG. 1. The curve is the hyperbola plotted from the formula, with 179 taken as the value of K; the crosses represent the results experimentally obtained.

The value of K being known, small percentages of CO in air could be ascertained by shaking the latter with a known volume of the blood and determining the Hb O_2 and Hb CO, the calculation being *per cent CO in air* $= \frac{21.9}{179} \times \frac{\text{Hb CO}}{\text{HbO}_2}$. In calculating the actual percentage of CO in the gas-sampling tube allowance was made for the amount of CO taken up by the blood with which the air was shaken. The colorimetric determination was

made by the method employed by Plesch (8), which is very simple and which gave fairly good results when used on blood with known percentages of CO. The principle is: Three samples of the same blood are saturated; No. 1 with air, No. 2 with CO, and No. 3 with the air containing the unknown percentage of CO. 0.05 cc. of each sample is diluted with 10 cc. of a 1 per cent solution of Na_2CO_3 in each of three small test-tubes of equal bore. No. 2 is added to No. 1 until the color is the same as in No. 3. If, for instance, equal color is obtained by adding 2.5 cc. of No. 2 to 5 cc. of No. 1, then the degree of saturation is $\frac{2.5}{2.5+5} = 33.3$ per cent.

Results.

In fourteen rabbits taken from the stock the blood volume was determined. In two of the female rabbits the values obtained were much higher than the average, and they later proved to be pregnant. They were placed in a separate group and the blood volume determined again, post partum. In some of the other rabbits the blood volume also was determined twice. The results are given in Tables I to V.

TABLE I.

Normal Male Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Blood volume.	Relation to body weight.	Cc. per 100 gm.
	1919	*gm.*	*per cent*	*cc.*		
77	July 3	1,500	(?)*	79.4	$\frac{1}{19.1}$	5.20
93	" 8	1,900	(?)*	95.7	$\frac{1}{19.9}$	5.02
89	" 10	1,600	93.4	80.9	$\frac{1}{19.8}$	5.05
80	" 10	2,250	94.3	107.1	$\frac{1}{21}$	4.76
99	" 14	3,010	86.2	146.9	$\frac{1}{20\ 5}$	4.88
82	" 14	2,200	87.7	115.4	$\frac{1}{19.06}$	5.25
98	" 15	2,200	93.4	111.1	$\frac{1}{19.8}$	5.05

* Determinations lost because of an incorrect hemoglobin standard.

TABLE II.

Normal Female Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Blood volume.	Relation to body weight.	Cc. per 100 gm.
	1919	*gm.*	*per cent*	*cc.*		
66	July 2	2,300	(?)*	122.2	1/18.8	5.30
76	" 7	1,250	83.3	50.8	1/24.6	4.06
88	" 9	1,960	86.2	100.6	1/19.4	5.14
83	" 11	2,630	76.9	129.2	1/20.3	4.93
87	" 11	2,320	51.4	127.5	1/18.2	5.49

* Determination lost because of an incorrect standard.

TABLE III.

Repeated Determinations.
Male Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Total oxygen capacity.	Blood volume.	Ratio.	Cc. per 100 gm.
	1919	*gm.*	*per cent*	*cc.*	*cc.*		
93	July 8	1,900	(?)*	(?)*	95.7	1/19.9	5.02
	" 21	1,940	59.3	10.14	92.4	1/21	4.76
77	" 3	1,500	(?)*	(?)*	79.4	1/19.1	5.20
	" 18	1,540	75.0	13.87	72.9	1/21.1	4.74
80	" 10	2,250	94.3	18.68	107.1	1/21.1	4.76
	" 17	2,270	90.9	17.54	102.1	1/22.2	4.50
99	" 14	3,010	86.2	21.08	146.9	1/20.5	4.88
	" 18	3,070	74.1	22.33	162.1	1/18.9	5.29

* Determination lost because of an incorrect standard.

TABLE IV.

Repeated Determinations.
Female Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Total oxygen capacity.	Blood volume.	Ratio.	Cc. per 100 gm.
	1919	*gm.*	*per cent*	*cc.*	*cc.*		
76	July 7	1,250	83.3	7.83	50.8	1/24.6	4.06
	" 15	1,350	66.6	8.30	66.8	1/20.2	4.95
81	" 11	2,320	51.4	12.12	127.5	1/18.2	5.49
	" 17	2,300	47.4	11.44	129.0	1/17.8	5.62

TABLE V.

Blood Volume in Pregnant Rabbits before and after Term.

Rabbit No.	Determination made.	Date.	Weight.	Hemoglobin	Total oxygen capacity.	Blood volume.	Ratio.	Cc. per 100 gm.	Remarks.
		1919	*gm.*	*per cent*	*cc.*	*cc.*			
65	Before.	July 8	2,650	69.4	22.86	177.3	1/14.9	6.70	Young ones, July 15.
	6 days post partum.	" 21	2,290	76.3	15.24	108.3	1/19.4	5.44	
87	Before.	" 9	2,560	94.0	24.29	141.1	1/18.1	5.52	
	"	" 16	2,570	74.4	22.2	161.3	1/15.3	6.54	Young ones, July 20.
	11 days after.	" 31	2,350	76.3	15.59	110.5	1/21.2	4.72	

In Tables III and IV is introduced a column, "total oxygen capacity," the figures in which indicate all the oxygen with which the blood is able to combine, calculated from the blood volume and the hemoglobin percentage. In our hemoglobin standard 100 per cent equals 18.5 volumes per cent of O_2.

DISCUSSION.

The results are expressed in parts of the crude body weight, though this may vary for various causes, such as food intake, content of bowels, etc. The average value for the blood volume in seven male rabbits (eleven determinations, Tables I and III) is $\frac{1}{20.21}$ of the body weight, or 4.95 cc. per 100 gm. The average in five non-pregnant female rabbits (seven determinations, Tables II and IV) is $\frac{1}{19.9}$ of the weight, or 5.02 cc. per 100 gm. If the two determinations from Table V in rabbits post partum are added, the mean for all seven females is the same, $\frac{1}{19.99}$, or 5.0 cc. per 100 gm. The results are in accordance with those obtained by Boycott (7) and coworkers with the washing out method.

The repeated determinations in Tables III and IV show a fairly close agreement in some of the rabbits, while in Rabbits 99 and 76 there is a considerable difference. But the total oxygen capacity is nearly constant in these two rabbits. Rabbit 76 had increased in weight from 1,250 to 1,350 gm., and to get comparable values the oxygen capacity in the first determination has to be multiplied by $\frac{1,350}{1,250} = 1.08$, and this gives the value of 8.46 cc. which is very close to the value found the second time, 8.30 cc. The same phenomenon is seen in the pregnant rabbit, No. 87, of Table V. The two determinations before partus gave $\frac{1}{18.4}$ and $\frac{1}{15.3}$ of the weight, which is a large increase in the blood volume. The hemoglobin concentration dropped, however, so that the oxygen capacity of the total blood supply of the animal remained constant, the blood merely having become diluted in the interval between determinations. About a week after partus the blood volume was again normal.

The blood volume of the rabbit, therefore, seems to be able to change normally, probably in the way that fluid passes in and out through the capillaries, a phenomenon in analogy with what has been observed in human beings in shock. Rabbits 99 (Table III), 76 (Table IV), and 87 (Table V), in which these changes in

the blood volume and constancy of the total oxygen capacity were so marked that they cannot be due to exper mental error, show the smallest volume the first time, when they were unexperienced and scared when put into the apparatus, and a larger volume the second time, when they were used to the procedure. The material is too small, however, to draw any conclusion on this point, but Douglas has observed the same phenomenon of changes in the volume and constancy of oxygen capacity.

Table V shows the well known fact that pregnant animals have a larger blood volume absolutely and relatively than non-pregnant. A week post partum the blood volume is restored to the normal value.

Blood Volume Determinations in Human Beings.

The arrangement of apparatus was nearly the same as that described by Haldane and Smith (4), except that an ordinary Wolff bottle, filled with sticks of potassium hydroxide,[1] was used for the absorption of the carbonic acid, as seen in Fig. 2, and the experiments lasted longer, the subjects breathing for 10 to 15 minutes after the entire amount of carbon monoxide was given. The oxygen was supplied from a cylinder as rapidly as was necessary in order to keep the amount of gas in the bag approximately constant. The estimation of the relative volume of air in the bag was facilitated by placing the bag horizontally with a scale behind it as an indicator of the degree of filling

The apparatus was filled with carbon monoxide to the three-way stop-cock before the experiment was started. The volume of the connecting parts and the bag, filled with air to the mark, was 3,700 cc. For the calculation of the amount of carbon monoxide left the volume of the lungs must be added; this is about 3,000 cc., and the total volume of the air in which the carbon monoxide was distributed thus was 6,700 cc.

The blood, being assumed to constitute not less than $\frac{1}{19}$ of the body weight, the amount of carbon monoxide given to the sub-

[1] Potassium hydroxide was used because of the high solubility of the potassium carbonate formed during the experiment; this carbonate was washed out by rinsing the sticks with water now and then.

ject was so calculated that the saturation of the blood with carbon monoxide would not exceed 20 to 25 per cent. For instance, with the body weight 70 kilos, hemoglobin 120 per cent, blood volume at least $\frac{70}{19}$ = 3.7 liters, and as 100 per cent hemoglobin =

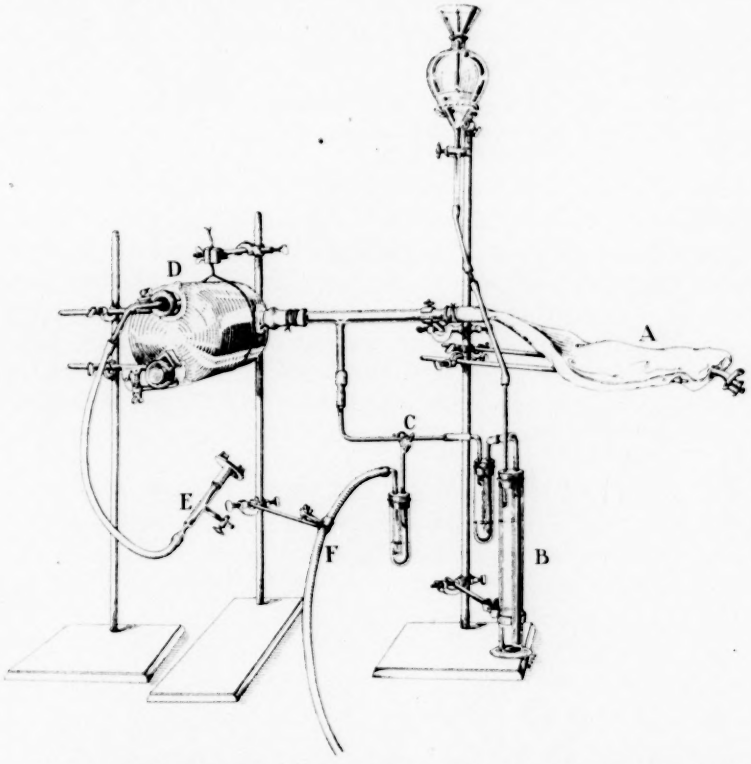

FIG. 2. A, rubber bag, B, volumetric measuring cylinder for carbon monoxide, C, three-way stop-cock, D, Wolff bottle for removing the carbon dioxide, E, mouthpiece, F, tubing connected with the oxygen cylinder.

18.5 volumes per cent of O_2 capacity, the capacity in this case would be 22.2 cc. per 100 cc. of blood; per 3,700 cc. it accordingly would be 821.4 cc. One, therefore, could safely give 164 cc. of carbon monoxide reduced to standard conditions (760 mm. and 0°).

The blood sample was drawn without stasis from the arm vein

and kept under paraffin oil. The carbon monoxide was determined by the method of Van Slyke and Salvesen,[2] 3 cc. being used for each determination, as the carbon monoxide content of the blood is rather small in these experiments.

The air in the bag was analyzed for oxygen, carbon dioxide, and carbon monoxide after each experiment, as in the animal experiments. In all except the last experiment the amount of carbon monoxide left was about 2 cc.: 1.47, 1.902, 2.53, 1.832, 1.96; average 1.938 cc. In the last experiment it was 4.42 cc., but in this case more oxygen was given than necessary, and the oxygen content of the bag was 33 per cent. This probably accounts for the slower absorption of the carbon monoxide, the dissociation curve of the carbon monoxide-hemoglobin being depressed when the oxygen percentage increases. If the experiments, therefore, are always performed in the same way, the amount of carbon monoxide left is constant, and if the apparatus has the same capacity as used in the present experiments, the correction which must be subtracted is 2 cc. The experiments done in this way are much simpler, the only determination which must be done being that of the carbon monoxide in the blood.

Six healthy individuals were examined, ranging in age from 23 to 37 years. The material was rather uniform as all were young people without any adipositas. The results are given in Table VI.

The average blood volume found was 3,888 cc., constituting $\frac{1}{16.8}$ of the weight, or 5.95 cc. per 100 gm. The extremes are $\frac{1}{14.3}$ and $\frac{1}{19.08}$. The largest volumes were found in Nos. 1 and 6, both of whom are tall and slim, especially No. 6 who is abnormally thin, and weighs much less than would correspond with his height. In No. 1 two determinations were made and the difference found is only 7 cc. of blood.

The mean value, then, is a little larger than that of Plesch, who found $\frac{1}{17.9}$ and smaller than that of Douglas, who found in two persons $\frac{1}{13.9}$ and $\frac{1}{12.5}$.

[2] Van Slyke, D. D., and Salvesen, H. A., *J. Biol. Chem.*, 1919, xl, 103.

All the persons experimented on felt comfortable, and did not have any disagreeable sensations. The breathing was easy, and even a certain degree of dyspnea is not likely to interfere with the use of this method in pathological cases. Former investigators

TABLE VI.

Blood Determinations in Human Beings.

No.	Date.	Name.	Age.	Weight.	Duration.	Hemoglobin.	Blood volume.		
								Ratio.	Cc. per 100 gm.
	1919		*yrs.*	*kg.*	*min.*	*per cent*	*cc.*		
1	July 28	Dr. H. S.	30	68.8	32	114	4,594	1/14.97	6.67
	Aug. 1	"		68.8	35	114	4,601	1/14.82	6.74
2	July 29	Mr. A. S.	26	60.9	28	124.9	3,464	1/17.6	5.68
3	" 29	Dr. H. A.	36	66.4	23	118	3,479	1/19.08	5.23
4	" 30	Dr. V. S.	37	72.7	27	113	3,877	1/18.7	5.35
5	" 31	Dr. J. T.	28	61	23	114	3,429	1/17.7	5.65
6	Aug. 1	Mr. R. T.	23	62.7	22	116	4,380	1/14.3	6.99
Average.				65.4			3,888	1/16.8	5.95

have used the carbon monoxide method in heart, kidney, and anemic cases without any difficulties.

It is hoped that the blood volume method with the easy technique for the carbon monoxide determination will be of more practical value than before.

SUMMARY.

Determinations of the blood volume by the carbon monoxide method with the simple technique previously described for the blood analysis have been made in fourteen rabbits and six normal men. Eleven determinations in seven male rabbits show an average blood volume of $\frac{1}{20.21}$ of the body weight, or 4.95 cc. per 100 gm. Nine determinations in five non-pregnant female rabbits show an average of $\frac{1}{19.99}$, or 5.0 cc. per 100 gm.

In two pregnant rabbits the blood volume was largely increased, absolutely and relatively; about a week post partum it was restored to normal again.

The blood volume of rabbits may change from time to time, but the total oxygen capacity remains constant.

Seven determinations in six healthy men show an average of $\frac{1}{16.8}$ of the body weight, or 5.95 cc. per 100 gm.

The author wishes to express his thanks to Dr. Donald D. Van Slyke on whose initiative this work was undertaken, and to Mr. Arthur H. Smith for his technical assistance during the experiments.

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A NOTE ON THE DETERMINATION OF CATALASE IN BLOOD.*

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During the course of several hundred determinations of catalase in the blood of normal and pathological individuals according to the method employed by Burge,[1] the author observed a consistent variation of from 15 to 35 per cent in the volume of oxygen evolved, depending upon the hydrogen peroxide solution used. The hydrogen peroxide used in these determinations was prepared by diluting commercial 3 per cent hydrogen peroxide solution with an equal volume of distilled water.

Method.

0.2 cc. of the blood was placed in a small crucible which was introduced into a bottle containing 100 cc. of approximately 1.5 per cent (4.6 volume per cent) hydrogen peroxide and a drop of caprylic alcohol. The bottle, which was kept at 22°C. in a water bath, was shaken 120 double shakes per minute for 10 minutes and the oxygen evolved was conducted through rubber tubes into inverted burettes and measured. The volume of oxygen obtained varied between 275 and 425 cc. with different bloods.

In a later paper Burge[2] states that he found it necessary to use the same make of hydrogen peroxide in all the determinations as the different makes gave different results. For his work he

* Published with permission of the Chief of the Laboratory, Lt. T. E. Buckman.

[1] Burge, W. E., *Am. J. Physiol.*, 1916, xli, 153.

[2] Burge, W. E., Kennedy, J., and Neill, A. J., *Am. J. Physiol.*, 1917, xliii, 435.

purchased 200 liters of hydrogen peroxide which was kept in a container in a dark, cool place.

It has been found, in this laboratory, that different samples of the same make of hydrogen peroxide often gave results which differed by 15 to 35 per cent. Since all the samples contained the same preservative (acetanilide) in approximately the same amount (0.05 per cent), it was assumed that the discrepancies were due to the difference in the acidity of the various samples.

A series of experiments was performed to test this assumption. A set of H_2O_2 solutions, 5.8 volumes per cent, containing varying amounts of HCl or NaOH was prepared. As alkaline solutions of hydrogen peroxide decompose on standing, the solutions for these experiments were freshly prepared.

2 cc. of blood containing a slight trace of potassium oxalate were diluted to 200 cc. with distilled water. 5 cc. of this solution, equivalent to 0.05 cc. of the blood, were placed in a small crucible and introduced into a bottle containing 20 cc. of hydrogen peroxide solution and one drop of caprylic alcohol. The bottle, kept at 22°C. in a water bath, was shaken 120 double shakes per minute for 10 minutes. The oxygen evolved was conducted through rubber tubes into inverted burettes and the volumes measured at intervals of $\frac{1}{2}$ minute. All gas volumes were calculated to standard conditions; *i.e.*, 0°C. and 760 mm. Hg. pressure.

The results are given in Charts 1 and 2.

A reference to the charts will show the effect of acid and alkali on the velocity of the reaction. The activity of the enzyme catalase was completely inhibited when the hydrogen ion concentration was pH 1, and was slight in a concentration of pH 2. The velocity of the reaction increases as the acidity of the hydrogen peroxide is decreased and is greatest when the solution is slightly alkaline. The activity of the enzyme was considerably retarded when the concentration of the NaOH was above pH 11.

Falk, McGuire, and Blount[3] point out that there is no well defined hydrogen ion concentration for maximum action with oxidase, peroxidase, and catalase. According to Sörensen,[4] the

[3] Falk, K. G., McGuire, G., and Blount, E., *J. Biol. Chem.*, 1919, xxxviii, 237.

[4] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 288.

optimum hydrogen ion concentration for catalase depends on the duration of the reaction. Thus he finds pH 6.73 the optimum for 320 minutes, and pH 7.1 for 40 minutes. With blood catalase under the experimental conditions stated above, I find the optimum to be about pH 7.5 for 10 minutes, about pH 8 for 5 minutes, and about pH 10 for 2 minutes.

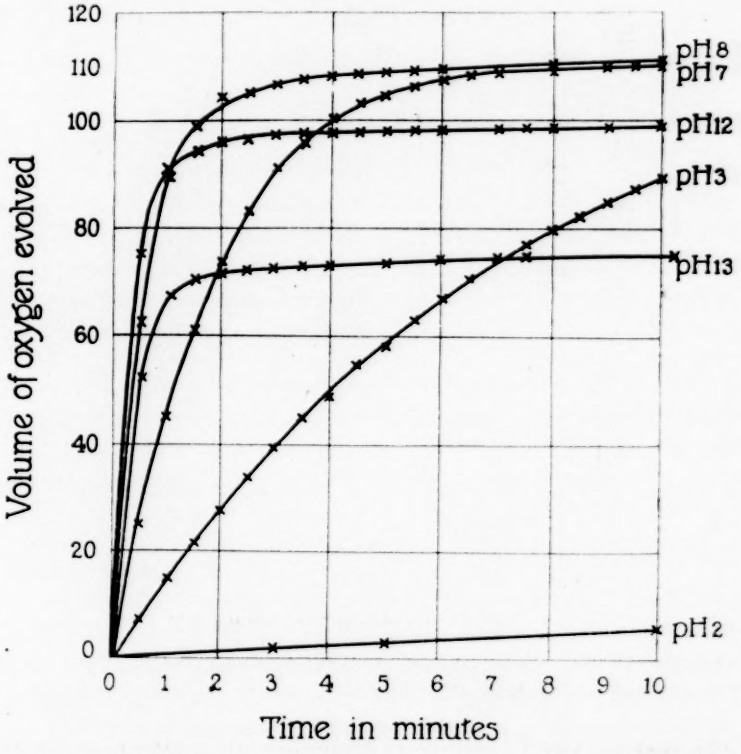

CHART 1. Curves showing the effect of the hydrogen ion concentration of the hydrogen peroxide on the velocity of the reaction between catalase and hydrogen peroxide.

As far as I am aware there is no satisfactory absolute method for the determination of catalase. Nearly all the methods in use give relative results. Slight changes in temperature, impurities in, and the reaction of the hydrogen peroxide influence the

reaction very materially. These experiments emphasize the caution which must be exercised in maintaining uniform conditions especially when a series of determinations is made over a long period of time.

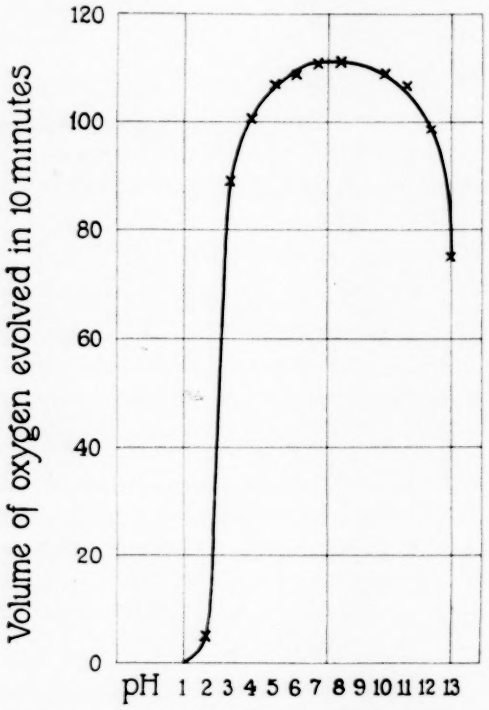

CHART 2. Curve showing the effect of the hydrogen ion concentration of the hydrogen peroxide on the volume of oxygen evolved in 10 minutes.

The author's thanks are due to Lieutenant T. E. Buckman and Lieutenant G. L. Foster of this laboratory for their helpful suggestions.

THE ACTION OF FURFUROL AND DEXTROSE ON AMINO-ACIDS AND PROTEIN HYDROLSATES.

By C. T. DOWELL and PAUL MENAUL.

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(Received for publication, September 22, 1919.)

While hydrolyzing the whole plant, including the seed, of some grain sorghums, we noticed that a large amount of furfurol was given off, and since we obtained an unusually large per cent of the nitrogen in the humin it occurred to us that this might be due to a reaction between the amino-acids and furfurol. A survey of the literature seems to confirm this idea. For example Grindley and Slater[1] found 15.79 per cent of the nitrogen in the humin formed in the hydrolysis of alfalfa hay, which was a much larger amount than that found in any other substance. Nollau[2] obtained 13.75 per cent of humin nitrogen from wheat bran. Hart and Sure[3] found 11.83 per cent of humin nitrogen when xylan was added in the hydrolysis of casein. Gortner[4] studied the action of formaldehyde, benzaldehyde, and furfurol on certain amino-acids and states that the humin nitrogen is probably due in great part to the formation of furfurol in the hydrolysis of the protein.

We found, in attempting to apply the Van Slyke method to analysis of the hydrolsate from the grain sorghum plant, not only a large amount of humin nitrogen but also incomplete precipitation of basic acids which was unexpected as 15 gm. of phosphotungstic acid had been added. The addition of 50 gm. of the acid was required for complete precipitations. This amount precipitated from 30 to 33 per cent of the total nitrogen. Only

[1] Grindley, H. S., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 2762.

[2] Nollau, E. H., *J. Biol. Chem.*, 1915, xxi, 611.

[3] Hart, E. B., and Sure, B., *J. Biol. Chem.*, 1916-17, xxviii, 241.

[4] Gortner, R. A., *J. Biol. Chem.*, 1916, xxvi, 177.

a small amount of the precipitate redissolved on warming, nor was it possible to decompose it except to a slight extent with ether-amyl-alcohol mixture. Grindley, Nollau, and others who have applied this method to feedstuffs do not mention having had such difficulties. However, Brewster and Alsberg[5] in an article which appeared after we had decided to discontinue our efforts to apply the method state that they obtained a much larger per cent of nitrogen in the phosphotungstic precipitate than should be obtained, and attributed this partly to the precipitation of the humin nitrogen by the phosphotungstic acid. They had the same difficulty as we in dissolving the precipitate. It seemed to us that this trouble was caused by the humin which remained in solution even after it was made alkaline, but was precipitated by the phosphotungstic acid.

These difficulties led us to study the action of furfurol and dextrose on amino-acids and protein hydrolsates. One would expect that some of the amino-acids would react more readily with aldehydes and sugars than would others, and if this is true it would make the application of the Van Slyke method to feedstuffs useless. The results obtained by McHargue[6] in hydrolyzing casein in the presence of starch for different lengths of time might be due to the difference in reactivity of the amino-acids with sugars or aldehydes. We have succeeded in obtaining but a few of the amino-acids. However, it is thought worth while to report the results already obtained and also our study of the action of furfurol and dextrose on amino-acids and protein hydrolsates.

EXPERIMENTAL.

Action of Furfurol on Amino-Acids.

Determination of the amino nitrogen was made by the Van Slyke method on the stock solution of the amino-acids and on the solution boiled with HCl and furfurol in acid solution. The results are shown in Table I. Two trials were made with glycine without getting evidence of a reaction between it and furfurol.

It was found that furfurol, when boiled with HCl, changes into a black humin-like mass and it was thought that the decrease in

[5] Brewster, J. F., and Alsberg, C. L., *J. Biol. Chem.*, 1919, xxxvii, 367.
[6] McHargue, J. S., *J. Agric. Research*, 1918, xii, 1.

TABLE I.

Action of Furfurol on Amino-Acids in 10 Per Cent HCl.

Acid.	Time boiled.	Amount of furfurol.	Volume of amino N_2 in 10 cc. of solution.	N_2 recovered.
	hrs.	*cc.*		*per cent*
Tyrosine	2	0	5.9	89.8
"	2	1	5.3	
"	2	0	27.0	
"	2	2	24.5	90.7
Cystine	2	0	32.5	
"	2	1	27.5	84.6
Glycine	2	0	33.7	100.0
"	2	1.5	33.5	

amino nitrogen might be due to adsorption by this substance. To determine whether or not this was true 50 gm. of grain sorghum plant, ground to pass through a 20 mesh, were hydrolyzed in an autoclave at 20 pounds pressure in 10 per cent HCl for 3 hours (we had shown this to give complete hydrolysis) and the humin was washed with 2,500 cc. of hot water by decantation. It was then washed in the same way with another 2,500 cc. and this last was evaporated to 25 cc. and the amino nitrogen determined; 2.14 mg. were obtained. The 18 gm. of humin contained 165.4 mg. of amino nitrogen. This with a similar result showed that our results with the amino-acids given in Table I were not due to adsorption.

Action of Furfurol on Protein Hydrolsates.

Samples of shrimp, casein, wool, and salmon were hydrolyzed in an autoclave as already described and, after filtering off the humin, portions of the solutions, which had been made neutral, were boiled in HCl solution with the addition of furfurol. The results are shown in Table II. The substances were selected so as to have hydrolsates containing largely basic acids in some solutions and mono-acids in others.

TABLE II.

Action of Furfurol on Protein Hydrolsates in Acid.

Hydrolsate.	Volume of amino N_2 in 10 cc. of solution before treatment with furfurol.	Amount of furfurol.	Concentration of acid.	Time of boiling.	Volume of amino N_2 in 10 cc. of solution after treatment with furfurol.	Amino N_2 recovered.
	cc.	*cc.*	*per cent*	*hrs.*	*cc.*	*per cent*
Shrimp	29.4	1	10	2	29.0	98.6
Casein	12.0	1	10	2	11.5	95.8
Wool	27.3	1	10	2	26.3	96.4
Salmon	39.0	1	10	2	36.3	93.0
Shrimp	16.4	2	5	16	13.1	79.8
Wool	17.4	2	5	16	13.5	77.5
Salmon	23.35	2	5	16	17.5	74.9

TABLE III.

Effect of Concentration of Acid on Reaction between Furfurol and Casein Hydrolsate.

Concentration of HCl.	Time of boiling.	Amount of furfurol.	Volume of amino N_2 in 10 cc. of solution before boiling.	Volume of amino N_2 after boiling.	Amino N_2 recovered.
per cent	*hrs.*	*cc.*			*per cent*
0	15	2	33.4	17.6	51.1
0	15	2	31.1	21.3	68.4
5	15	2	30.2	26.3	87.0
10	15	2	30.2	27.3	90.3
20	15	2	16.3	15.7	96.3

TABLE IV.

Effect of Concentration of Acid on Reaction between Dextrose and Casein Hydrolsates.

Concentration of HCl.	Time of boiling.	Concentration of Dextrose.	Volume of amino N_2 in 10 cc. of solution before boiling.	Volume of amino N_2 after boiling.	Amino N_2 recovered.
per cent	*hrs.*	*per cent*			*per cent*
20	15	4	33.4	33.4	100.0
20	15	4	31.1	31.1	100.0
20	15	4	16.3	16.0	98.1
10	15	4	30.2	28.9	95.3
5	15	4	30.2	28.8	95.3
Neutral.	15	4	33.4	28.1	84.1
"	15	4	31.1	26.0	83.6

TABLE V.

Effect of Boiling Casein Hydrolsate in 20 Per Cent HCl for 15 Hours in the Presence of Arabinose, Starch, Gum Arabic, and Cellulose.

Name of substance.	Amount of substance.	Volume of amino N_2 in 10 cc. of solution before boiling.	Volume of amino N_2 after boiling.	Amino N_2 recovered.
	gm.			*per cent*
Arabinose	1	16.3	15.4	94.4
Gum Arabic	2	16.3	15.9	97.5
Starch	2	16.3	15.8	96.9
Cellulose	2	16.3	15.8	96.9

SUMMARY.

1. Glycine does not react with furfurol. There is no explanation of this since tyrosine and cystine give decided evidence of reaction.

2. It is shown that but a slight amount of the humin nitrogen is due to adsorption.

3. The hydrolsates of shrimp, casein, wool, and salmon react with furfurol.

4. The greatest decrease in amino N_2 is obtained when the reaction between a protein hydrolsate and furfurol takes place in a neutral or slightly acid solution. This is probably due to the fact that furfurol forms a humin-like mass when boiled in acid solution.

5. The greatest effect is obtained in the reaction between dextrose and a protein hydrolsate when the solution is either neutral or slightly acid. This shows that dextrose reacts directly with the amino-acids and not through the intermediate formation of furfurol as we thought might be true.

Our results with dextrose together with the fact that we seemed to get a precipitation of humin nitrogen with phosphotungstic acid leads us to think that the method proposed recently by Eckstein and Grindley[7] for the analysis of feedstuffs by the Van Slyke method would lead to low results. In order to remove the carbohydrates, they digest the sample for 60 hours in 0.1 per cent

[7] Eckstein, H. C., and Grindley, H. S., *J. Biol. Chem.*, 1919, xxxvii, 373.

hydrochloric acid. This converts starches to sugars, and since the acid concentration is low, the conditions are most favorable for the reaction of the sugars with the amino-acids. It is true that this concentration of acid would not hydrolyze much of the protein, but it should be remembered that there is a high per cent (30 to 40 per cent) of soluble nitrogen, mostly in the form of amino-acids, in many feedstuffs.

6. The results given in Table V show, as would be expected, that substances which yield either sugars or furfurol when boiled with hydrochloric acid decrease the amino nitrogen when boiled with a protein hydrolsate.

7. It was found that complete hydrolysis of various proteins is brought about by heating in 10 per cent HCl in an autoclave for 3 hours at a pressure of 20 pounds.

THE HEAT COAGULATION OF MILK.*

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(Received for publication, September 17, 1919.)

The coagulation of milk by heat was first observed by Hammersten,[1] who found that it occurred at from 130–150°C. with different samples of milk. Since then the question has been studied very little, and no tenable explanation has ever been given for the difference in the coagulating points of milks from different cows. In recent years a knowledge of the factors which determine this difference has become very desirable, for these same factors undoubtedly determine whether a condensed milk will coagulate when it is sterilized. The coagulation of condensed milk on sterilizing causes serious losses in the milk-condensing industry.

In the manufacture of condensed milk, the fresh milk is first pasteurized or "preheated" at from 180–210°F. for from 1 to 20 minutes. The condensing is done under vacuum at 130–160°F. After the desired concentration has been attained, the milk is put into cans, sealed, and then sterilized at 224–240°F. for 20 to 50 minutes. It is during the sterilizing process that the coagulation occurs. Because it occurs so frequently, all the condensed milk is placed into shaking machines to break up any loose coagulum that may have formed. However, frequently the coagulum is so firm that even after shaking the milk remains lumpy. Such a product is rejected by the consuming public, and thus is a loss.

Manufacturers have sought to solve the problem by controlling the acidity of the milk. They have set an arbitrary standard such as 0.18 per cent acid (calculated as lactic acid), above which

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Quoted from literature referred to in, Kastle, J. H., Chemistry of milk, *Hyg. Lab., Bull. 56*, 1909.

they reject all milk. This has led to much difficulty, because often, immediately after it is drawn from the cow, milk has a higher titratable acidity than 0.18 per cent. Thus the condenseries may be rejecting perfectly fresh milk, believing that they are remedying their difficulty in this way, although it has never been demonstrated that titratable acidity is related to the coagulation. The factors involved in the coagulation have never been determined, and no explanation is available on which to base a remedy for this difficulty.

To offer an explanation for the difference in the coagulating points of different milk samples the following factors were studied: titratable acidity, hydrogen ion concentration, concentration of the milk, and composition and balance of the milk salts.

The Heat Test.

The temperature at which the milk was heated was arbitrarily set at 136°C. At first the heating was done in an autoclave at 50 pounds pressure for 20 minutes, and in that way the milks were differentiated into coagulating and non-coagulating. With the autoclave, it took about 10 minutes to get up to the desired pressure; and, after the milk had been heated, the pressure had to be released gradually to prevent the milk from boiling over. The disadvantages of this method were such that there were no sharp limits from which to calculate the 20 minute interval, and it was impossible to determine the relative rates at which the milk samples coagulated.

To overcome these disadvantages the milk was placed into small glass tubes, sealed, and then heated in a xylene vapor bath which was constant at 136°C. within 0.5°C. The sealed tubes were clamped in a rack, so arranged that it could be tilted to invert the tubes, to see how the milk would flow, and in that way it was possible to determine the exact length of time required for each sample to coagulate. The milk in the sealed tubes was up to 136°C. in less than 1 minute, so the point from which to calculate the time was practically the instant the tubes were inserted into the vapor bath.

Titratable Acidity.

Since condenseries are attempting to remedy the coagulation problem by rejecting milk above 0.18 per cent acid (calculated as

TABLE I.

Titratable Acidity and Coagulation.

May 8, 1919.			May 10, 1919.			May 16, 1919.		
Cow No.	Titratable acidity.	Coagulation.	Cow No.	Titratable acidity.	Coagulation.	Cow No.	Titratable acidity.	Coagulation.
	Lactic acid.			Lactic acid.			Lactic acid.	
	per cent	*min.*		*per cent*	*min.*		*per cent*	*min.*
1	0.257	20—*	1	0.241	6	31	0.203	4½
2	0.235	3	2	0.231	11	2	0.193	20—
3	0.216	20—	26	0.228	5	1	0.192	20—
4	0.214	20—	31	0.222	5½	3	0.188	4½
5	0.210	8	27	0.212	6	27	0.188	6½
6	0.207	20—	3	0.212	5½	6	0.188	20—
7	0.206	6	4	0.211	20—	4	0.188	20—
28	0.201	20—	5	0.205	5½	28	0.186	20—
9	0.200	20—	9	0.197	20—	12	0.184	10
10	0.200	20—	28	0.199	20—	26	0.184	3
11	0.195	4½	6	0.195	20—	5	0.183	6½
12	0.191	20—	29	0.192	5½	9	0.182	20—
13	0.190	20—	10	0.190	20—	14	0.182	9
14	0.189	6½	13	0.186	9	29	0.182	1¾
15	0.182	20—	12	0.185	11	11	0.178	3½
16	0.179	4	11	0.184	5	13	0.175	4
8	0.174	9	8	0.174	20—	10	0.171	20—
17	0.172	20—	14	0.175	6½	30	0.165	20—
18	0.167	20—	15	0.172	20—	15	0.163	20—
19	0.158	12	17	0.166	20—	8	0.163	6½
20	0.157	20—	18	0.160	20—	17	0.156	20—
21	0.156	20—	30	0.162	4	18	0.154	20—
22	0.148	3	7	0.162	2	7	0.148	2
23	0.146	3½	21	0.157	20—	20	0.143	20—
24	0.143	20—	20	0.147	20—	19	0.135	20—
25	0.120	2	23	0.145	5½	22	0.133	2
			19	0.144	20—	23	0.130	4
			22	0.144	2	24	0.128	20—
			24	0.141	20	21	0.128	20—
			25	0.131	1½	25	0.102	1½

*20— = no coagulation in 20 minutes.

lactic acid), it was of interest to know how much variation there was in the tritratable acidity of milk from individual cows, and what relation the acidity would bear to the coagulation. To study this, samples were taken from the University herd and titrated immediately, and the heat test in the xylene vapor bath applied as soon as possible. The results given in Table I were obtained.

The titratable acidity varies from 0.102 to 0.257 per cent. Out of the 86 samples, 45 are above 0.18 per cent.

In fresh milk there is no direct relation between titratable acidity and coagulation, as is evident from Table II. If fresh milk samples were more nearly alike in titratable acidity, then titratable acidity might bear a direct relationship to the heat

TABLE II.

Summary of Titratable Acidity and Coagulation.

Date.	No. of samples.	No. above 0.18 per cent acid.*	No. that are 20 +.†	No. below 0.18 per cent acid.‡	No. that are 20 +.
May 8.....	26	15	5	11	6
" 10.....	30	14	7	16	7
" 16.....	30	16	11	14	6
Total.....	86	45	23	41	19

* Per cent above 0.18 per cent acidity, coagulating 20+ = 51.2 per cent.

† 20+ means coagulation within 20 minutes.

‡ Per cent below 0.18 per cent acidity, coagulating 20+ = 46.4 per cent.

coagulation of commercial milk samples. The acidity would then be a measure of the amount of fermentation that had taken place. Lactic acid fermentation lowers the coagulating point in two ways; (1) it changes the reaction, and (2) it lowers the citric acid content of the milk very rapidly.[2] Both of these are factors in lowering the coagulating point, as will be shown later.

Since fresh milk samples vary so widely in titratable acidity, it is impossible to measure the extent of acid fermentation in a sample by titration. For this reason it is impossible to use titratable acidity as a criterion of coagulability.

[2] Bosworth, A. W., and Prucha, M. J., *Tech. Bull. 14, N. Y. Agric. Exp. Station*, 1910.

TABLE III.

Hydrogen Ion Concentration and Coagulation.

Date.	Cow.	pH	C_H	Coagulation in 20 min.
1919				
Feb. 21	31	6.55	2.82×10^{-7}	+++*
	32	6.83	1.48×10^{-7}	+
	13	6.58	2.63×10^{-7}	+++
	24	6.83	1.48×10^{-7}	−
" 25	13	6.25	5.62×10^{-7}	−
	32	6.66	2.19×10^{-7}	−
	31	6.66	2.19×10^{-7}	+++
" 26	13	6.58	2.63×10^{-7}	+++
	32	6.69	2.04×10^{-7}	++
	31	6.70	1.99×10^{-7}	++
	24	6.73	1.86×10^{-7}	+
	14	6.70	1.99×10^{-7}	+++
" 27	13	6.44	3.62×10^{-7}	+
	32	6.64	2.29×10^{-7}	++
	31	6.64	2.29×10^{-7}	+++
" 27	24	6.70	1.99×10^{-7}	−
	14	6.44	3.62×10^{-7}	−
	33	6.59	2.56×10^{-7}	+++
" 28	13	6.50	3.16×10^{-7}	−
	31	6.94	1.15×10^{-7}	+++
	32	6.92	1.20×10^{-7}	−
	24	6.93	$1.17\times10-$	++
	14	6.50	3.16×10^{-7}	−
	33	6.68	2.09×10^{-7}	+++
Mar. 3	13	6.67	2.14×10^{-7}	−
	31	6.84	1.44×10^{-7}	−
	33	6.69	2.04×10^{-7}	++
" 5	33	6.64	2.29×10^{-7}	+++
	13	6.64	2.29×10^{-7}	−
	31	6.93	1.17×10^{-7}	+
	32	6.79	1.62×10^{-7}	−
	24	6.79	1.62×10^{-7}	−
	14	6.58	2.63×10^{-7}	−
" 6	24	6.97	1.07×10^{-7}	+
	33	6.59	2.57×10^{-7}	+++
" 7	31	6.85	1.41×10^{-7}	+++
" 10	33	6.79	1.62×10^{-7}	+++

* Number of plus signs indicates degree of firmness.

Hydrogen Ion Concentration.

Titratable acidity does not give an index to true acidity, or hydrogen ion concentration, so that if there is any relation between acidity and coagulation, it would be most likely to exist between the hydrogen ion concentration and coagulation. To study this possibility the hydrogen ion concentration of fresh milk was determined by means of the gas chain method, and the heat test was applied by means of the autoclave. The results given in Table III were obtained.

From a study of the data it becomes evident that the hydrogen ion concentration is not the determining factor in the coagulation. Samples of equal C_H do not always respond alike to the heat test; one may remain liquid, and the other may form a firm coagulum. In a large number of cases samples of high C_H did not coagulate, whereas samples of lower C_H did, the exact reverse of what should happen if true acidity was the cause of the coagulation.

We must conclude from this that in fresh milk C_H is not the determining factor in the coagulation. However, it may become a factor, for if we change the reaction of a milk sample by adding small amounts of acids the coagulating point is lowered.

Concentration.

The concentration of the milk would be expected to influence the coagulating point. This was found to be the case when milk was diluted (Table IV).

TABLE IV.

Relation of Coagulation to Concentration.

25 cc. of milk + H_2O.	Coagulation time.
H_2O added.	
cc.	*min.*
0.0	$1\frac{1}{2}$
1.0	2
2.0	$2\frac{3}{4}$
3.0	14
4.0	35–
5.0	35–
6.0	35–

Not only the concentration of the casein influences the coagulating point, but also the concentration of the serum. This was determined by comparing the effect of water dilution to the effect of dilution with milk serum obtained by filtering the milk through Pasteur-Chamberlain filters (Table V).

In the dilution with water, where the casein and the serum are both diluted, the effect is greater than where the casein alone is diluted by adding serum; therefore, the concentration of the serum is also a factor influencing the coagulating point.

Concentration of casein and of serum may in part explain the difference in the coagulating points of different milk samples.

TABLE V.

Relation of Coagulation to Concentration of Serum.

25 cc. of milk + serum.	Coagulation time.
Serum added.	
cc.	*min.*
0.0	$1\frac{1}{2}$
0.1	$1\frac{3}{4}$
0.2	2
25 cc. of milk + H_2O.	Coagulation time.
H_2O added.	
cc.	*min.*
0.0	$1\frac{1}{2}$
0.1	$2\frac{1}{2}$
0.2	4

However, in most cases, with the slight variation in concentration, this factor is of minor importance, just as C_H is. There must be another factor of greater importance.

Composition and Balance of Milk Salts.

Since electrolytes have a very marked effect upon the stability of colloids, we should expect that variations in the salt composition would influence the stability of the casein in the milk.

That the various salts exert an influence on the coagulating point was shown in a number of cases.

The effect of an addition of ammonium oxalate to milk that previously coagulated is shown in Table VI.

The removal of calcium by precipitation prevents coagulation in most cases and similarly in most cases the addition of small amounts of calcium salts lowers the coagulating point. This coagulation can again be balanced by means of sodium citrate or dipotassium phosphate (Tables VII, VIII, IX, and X). Coagulation caused by $MgCl_2$ or $BaCl_2$ can also be balanced by sodium citrate (Tables XI and XII).

In most cases coagulation can be prevented by the addition of citrates or phosphates, the coagulation being due to an excess of calcium and magnesium. However, in a few cases the addition of citrates or phosphates did not prevent coagulation, but rather

TABLE VI.

Ammonium Oxalate Prevents Coagulation.

5 cc. of milk + 10 per cent $(NH_4)_2C_2O_4$. $(NH_4)_2C_2O_4$ added.	Coagulation in 20 min.
drops	
0	+++
1	++
2	+
3	−
4	−−

hastened it. In these cases the addition of the proper amount of calcium salts prevents coagulation or at least raises the coagulating point (Tables XIII and XIV).

From the data we see that the calcium and magnesium are balanced by the phosphates and citrates of the milk practically in gram-equivalent amounts. The sodium and potassium chlorides in the concentrations present do not have any marked influence on the coagulating point, so that the balance of the four constituents, calcium, magnesium, citrates, and phosphates, largely determines whether a milk will coagulate or not. If calcium and magnesium are in excess, the milk will coagulate on heating. If calcium and magnesium are properly balanced with the phosphates and citrates, the optimum stability obtains. If phosphates and citrates are in excess, coagulation will also result.

TABLE VII.

*Balance between Calcium and Citrates.**

25 cc. of milk plus.			Coagulation time.
M/2 Ca acetate.	M/2 Na citrate.	H_2O	
cc.	*cc.*	*cc.*	*min.*
0.0	0.0	1.3	3
0.3	0.0	1.0	$\frac{1}{2}$
0.3	0.1	0.9	$2\frac{1}{2}$
0.3	0.2	0.8	3
0.3	0.3	0.7	2
0.3	0.4	0.6	$1\frac{1}{2}$

* The sodium citrate consisted of 25 cc. of sodium M/2 citrate plus 3 cc. of M/2 citric acid. This solution was distinctly acid, so that the balancing effect could not have been due to neutralization of acidity by means of the sodium citrate.

TABLE VIII.

*Balance between Calcium and Citrates.**

25 cc. of milk plus.			Coagulation time.
M/2 Ca acetate.	M/2 Na citrate.	H_2O	
cc.	*cc.*	*cc.*	*min.*
0.0	0.0	1.6	4
0.4	0.0	1.2	$\frac{1}{2}$
0.4	0.2	1.0	40—
0.4	0.4	0.8	40—
0.4	0.6	0.6	$2\frac{1}{4}$
0.4	0.8	0.4	2

* The sodium citrate consisted of 25 cc. of M/2 sodium citrate plus 1 cc. of M/2 citric acid.

TABLE IX.

Balance between Calcium and Citrates.

25 cc. of milk plus.			Coagulation time.
M/2 Ca acetate.	M/2 Na citrate.	H_2O	
cc.	*cc.*	*cc.*	*min.*
0.0	0.0	1.8	25—
0.8	0.0	1.0	$\frac{1}{8}$
0.8	0.4	0.6	$\frac{1}{8}$
0.8	0.6	0.4	25—
0.8	0.8	0.2	25—
0.8	1.0	0.0	$4\frac{1}{2}$

TABLE X.

Balance between Calcium and Phosphates.

25 cc. of milk plus.			Coagulation time.
M/2 Ca acetate.	M/2 K_2HPO_4	H_2O	
cc.	*cc.*	*cc.*	*min.*
0.0	0.0	1.2	20—
0.5	0.0	0.7	$\frac{1}{4}$
0.5	0.2	0.5	$\frac{3}{8}$
0.5	0.3	0.4	1
0.5	0.4	0.3	20—
0.5	0.5	0.2	20—
0.5	0.6	0.1	20—
0.5	0.7	0.0	6

TABLE XI.

Balance between Magnesium and Citrates.

25 cc. of milk plus.			Coagulation time.
M/2 $MgCl_2$	M/2 Na citrate.	H_2O	
cc.	*cc.*	*cc.*	*min.*
0.0	0.0	0.7	20—
0.3	0.0	0.4	$\frac{1}{2}$
0.3	0.2	0.2	20—
0.3	0.3	0.1	20—
0.3	0.4	0.0	8

TABLE XII.

Balance between Barium and Citrates.

25 cc. of milk plus.			Coagulation time.
M/2 $BaCl_2$	M/2 Na citrate.	H_2O	
cc.	*cc.*	*cc.*	*min.*
0.0	0.0	0.4	20—
0.2	0.0	0.2	$\frac{5}{8}$
0.2	0.2	0.0	20—

Thus the coagulation of a milk sample on heating may be due either to an excess or a deficiency of calcium and magnesium. We may explain this in the following manner. The casein of the milk is most stable with regard to heat coagulation when it is in combination with a definite amount of calcium. If the calcium

combined with the casein is above or below this optimum, the casein is not in its most stable condition. The calcium in the milk distributes itself between the casein, citrates, and phosphates chiefly. If the milk is high in citrate and phosphate content, more calcium is necessary in order that the casein may retain its optimum calcium content after competing with the citrates and phosphates. If the milk is high in calcium, there may not be

TABLE XIII.

A Sample in Which Calcium Prevents Coagulation.

25 cc. of milk plus.			Coagulation time.
M/2 Ca acetate.	M/2 Na citrate.	H_2O	
cc.	cc.	cc.	min.
0.0	0.0	0.8	$1\frac{1}{2}$
0.2	0.0	0.6	20—
0.2	0.1	0.5	$1\frac{1}{4}$
0.2	0.2	0.4	1
0.2	0.3	0.3	$\frac{3}{4}$
0.2	0.4	0.2	$\frac{3}{4}$

TABLE XIV.

A Sample in Which Calcium Raises the Coagulating Point.

25 cc. of milk plus.		Coagulation time.
M/4 Ca acetate.	H_2O	
cc.	cc.	min.
0.0	0.5	$1\frac{3}{4}$
0.1	0.4	2
0.2	0.3	$2\frac{1}{4}$
0.3	0.2	3
0.4	0.1	6
0.5	0.0	$2\frac{1}{2}$

sufficient citrate and phosphate to compete with the casein to lower its calcium content to the optimum. In such a case the addition of citrates or phosphates makes the casein more stable by reducing its calcium content. The magnesium functions by replacing the calcium in the citrates and phosphates.

In most cases the coagulation is due to an excess of calcium and magnesium. It is possible to balance this excess by citrates,

TABLE XV.

Analyses to Show Balance between Salts as Related to Coagulation.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sample No.	Citric acid.	P_2O_5	CaO	MgO	Citric acid Gram-equivalent.	P_2O_5 Gram-equivalent.	CaO Gram-equivalent.	MgO Gram-equivalent.	Citric acid + P_2O_5 Gram-equivalent.	CaO + MgO Gram-equivalent.	Column 11 minus Column 10.	Column 12 minus 0.40.	Coagulation time.	Total solids.
	per cent	*per cent*	*per cent*	*per cent*									*min.*	*per cent*
1	0.100	0.216	0.189	0.023	0.52	1.94	3.38	0.56	2.46	3.94	+1.48	+1.08	1½	12.41
2	0.292	0.255	0.194	0.021	1.52	2.30	3.41	0.51	3.82	3.98	+0.16	−0.24	1¾	15.02
3	0.191	0.236	0.184	0.025	0.99	2.13	3.29	0.62	3.12	3.91	+0.79	+0.39	2	13.55
4	0.130	0.193	0.148	0.022	0.68	1.74	2.64	0.55	2.42	3.19	+0.77	+0.37	2	9.79
5	0.165	0.292	0.189	0.022	0.86	2.63	3.38	0.55	3.49	3.93	+0.44	+0.04	3	15.20
6	0.145	0.277	0.210	0.021	0.76	2.50	3.75	0.53	3.26	4.28	+1.02	+0.62	3½	15.25
7	0.220	0.237	0.185	0.028	1.15	2.13	3.30	0.70	3.28	4.00	+0.72	+0.32	4	14.40
8	0.240	0.200	0.179	0.024	1.25	1.80	3.19	0.60	3.05	3.79	+0.74	+0.34	4	13.19
9	0.220	0.294	0.181	0.029	1.15	2.65	3.23	0.74	3.80	3.97	+0.17	−0.23	4½	14.30
10	0.207	0.280	0.204	0.025	1.08	2.52	3.64	0.63	3.60	4.27	+0.67	+0.27	4½	14.76
11	0.213	0.287	0.196	0.027	1.11	2.58	3.50	0.67	3.69	4.17	+0.48	+0.08	6½	14.14
12	0.164	0.278	0.190	0.026	0.85	2.49	3.39	0.65	3.34	4.04	+0.70	+0.30	6½	14.98
13	0.163	0.233	0.184	0.026	0.85	2.10	3.28	0.64	2.95	3.92	+0.87	+0.47	6½	14.02
14	0.124	0.258	0.170	0.025	0.65	2.32	3.04	0.62	2.97	3.66	+0.69	+0.29	9	14.14
15	0.312	0.246	0.204	0.023	1.62	2.21	3.64	0.59	3.83	4.24	+0.41	+0.01	10	13.23
16	0.194	0.273	0.158	0.024	1.01	2.46	2.82	0.60	3.47	3.42	−0.05	−0.45	20−	13.34
17	0.185	0.252	0.167	0.021	0.96	2.25	2.98	0.52	3.21	3.50	+0.29	−0.11	20−	13.75
18	0.168	0.262	0.164	0.026	0.88	2.36	2.93	0.65	3.24	3.58	+0.34	−0.06	20−	12.47

19	0.192	0.234	0.160	0.021	1.00	2.11	2.86	0.53	3.11	3.39	+0.28	−0.12	20−	12.61
20	0.210	0.249	0.193	0.025	1.09	2.24	3.45	0.61	3.33	4.06	+0.67	+0.27	20−	12.65
21	0.234	0.240	0.132	0.026	1.22	2.16	2.36	0.64	3.38	3.00	−0.38	−0.78	20−	14.17
22	0.213	0.196	0.145	0.019	1.11	1.76	2.59	0.46	2.87	3.05	+0.18	−0.22	20−	10.81
23	0.237	0.253	0.176	0.021	1.23	2.28	3.14	0.52	3.51	3.66	+0.15	−0.25	20−	13.41
24	0.225	0.240	0.158	0.022	1.17	2.16	2.82	0.55	3.33	3.37	+0.04	−0.36	20−	14.09
25	0.170	0.238	0.148	0.025	0.89	2.14	2.64	0.62	3.03	3.26	+0.33	−0.07	20−	14.39
26	0.243	0.196	0.164	0.023	1.27	1.77	2.93	0.57	3.04	3.50	+0.46	+0.06	20−	11.34
27	0.216	0.219	0.156	0.022	1.13	1.97	2.79	0.55	3.10	3.34	+0.24	−0.16	20−	12.59
28	0.238	0.291	0.162	0.022	1.24	2.62	2.89	0.54	3.86	3.43	−0.43	−0.83	20−	12.67
29	0.161	0.224	0.179	0.020	0.84	2.02	3.19	0.49	2.86	3.68	+0.82	+0.42	20−	13.60
30	0.144	0.206	0.139	0.020	0.75	1.85	2.48	0.51	2.60	2.99	+0.39	−0.01	20−	11.36

phosphates, carbonates, and other salts. It is also stated that danger of coagulation may be avoided in the actual practice of condensing milk by lengthening the "preheating" period, using higher temperatures. This may have the effect of lowering the soluble calcium content by precipitating part of it as insoluble calcium phosphate.[1]

To demonstrate the importance of the salt balance in the coagulation of milk, a number of samples were analyzed for total citric acid, phosphorus, calcium, and magnesium (Columns 2, 3, 4, and 5, Table XV). To calculate the balance between citric acid and phosphates, and calcium and magnesium the percentages were converted into gram-equivalents as follows:

$$(a)\ \frac{\text{Citric acid}}{192} \times 100$$

$$(b)\ \frac{P_2O_5 \times 100}{71} \times \frac{7}{11}\ *$$

$$(c)\ \frac{CaO \times 100}{56}$$

$$(d)\ \frac{MgO \times 100}{40}$$

* Multiply by $\frac{7}{11}$ because at pH 6.50, the average reaction of milk, the ratio of primary to secondary phosphate, is such that the mean basicity of the phosphates is approximately $\frac{7}{11}$ of what it would be if all the phosphates were secondary phosphates.

Column 10 shows the sum of citric and phosphoric acids in gram-equivalents; Column 11, the sum of calcium and magnesium in gram-equivalents. Column 12 shows the balance; a plus sign showing an excess of calcium and magnesium, and a minus sign showing an excess of citric and phosphoric acids. In only a few cases is there an excess of citric and phosphoric acids, and the excess is small. Those that coagulated had the largest excess of calcium and magnesium. To make this result more apparent, Column 13 shows the values of Column 12 minus 0.40. This figure was arbitrarily chosen and subtracted so as to make the coagulating samples have a plus sign and the non-coagulating samples have a minus sign. In five cases out of the thirty this result does not hold. However, the fact that, in twenty-five out of the thirty samples, those having the highest excess of calcium and magnesium

over citrates and phosphates coagulated and those having the lowest excess did not coagulate, indicates that this factor is very important.

The five exceptions may be due to the other factors, concentration and reaction. Samples 2 and 9, with their small excesses of calcium and magnesium, should not coagulate; however, both samples are high in total solids. Samples 20 and 26 did not coagulate although the excess of calcium and magnesium is high; again the explanation may lie partly in the concentration of the milk, both samples being low in total solids. If the pH had been determined we might have gained further insight into these exceptions and an explanation for the irregularity of Sample 29.

SUMMARY AND CONCLUSIONS.

1. The main factor in the heat coagulation of fresh milk is the composition of the milk salts. Apparently casein requires a definite optimum calcium content for its maximum stability. The calcium content of casein is largely controlled by the magnesium, citrates, and phosphates present.

2. In fresh milk there is no relation between titratable acidity and heat coagulation.

3. Acid fermentation in milk lowers the coagulating point by changing the reaction and by lowering the citric acid content. However, the titratable acidity of fresh milk samples varies so widely that it is impossible to determine the extent of acid fermentation by titration. Therefore it is impossible to use the acidity of milk as a criterion of coagulability.

4. Difference in concentration accounts partly for the difference in coagulation of fresh milk samples.

5. Hydrogen ion concentration is not the determining factor in fresh milk coagulation. It is nevertheless a factor in fresh milks, and in commercial milks it may become an important factor.

THE ACTION OF INTRAVENOUS INJECTIONS OF PANCREAS EMULSIONS IN EXPERIMENTAL DIABETES.

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It is evident that the demonstration of a beneficial effect of a pancreas preparation, when administered parenterally to a diabetic animal, would be of importance both theoretically and practically. Theoretically it would support the internal secretion hypothesis of the origin of diabetes. Practically it would suggest a possible therapeutic application.

The early work in this field was either negative or, as Pflüger,[1] and Leschke[2] have shown, is open to serious criticism. The usual criterion was a lowering of the concentration of sugar in the urine or a diminution of the total 24 hour output of sugar. Either of these effects may easily be caused by a diminished intake of food, due to the loss of appetite usually resulting from the treatment, to a change in the character of the diet, or to an influence on the kidneys. In clinical experience, the *feeding* of pancreas preparations has sometimes seemed to have a favorable subjective effect but with no constant antidiabetic action. Often this appeared to be due to supplying the *external* secretion to patients in need of it. Parenteral administration usually has not given good results. In a brief résumé of the literature Allen[3] says: "Though pancreas feeding may have at least a digestive value in some cases of diabetes, injections of pancreatic preparations have proved both useless and harmful. The failure began with Minkowski, and has continued to the present without an exception."

The more recent work may be summarized very briefly. Scott[4] found that intravenous injections of water extracts of pancreatic tissue, which had previously been extracted with alcohol, diminished temporarily the

[1] Pflüger, E., *Arch. ges. Physiol.*, 1907, cxviii, 267.

[2] Leschke, E., *Arch. Physiol.*, 1910, 401.

[3] Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 815.

[4] Scott, E. L., *Am. J. Physiol.*, 1911–12, xxix, 306.

sugar excretion of diabetic dogs and lowered their D/N ratio. Murlin and Kramer[5] injected boiled pancreatic extracts into depancreatized dogs and found a temporary reduction in the output of sugar. A mixed boiled extract of pancreas and duodenal mucosa produced a greater fall, and in one case a complete disappearance of the urinary sugar. The results were referred by these authors mainly to the sodium carbonate present in the extracts, because Ringer's solution, which had been brought to about the same degree of alkalinity as the medium used for the extract, was found to have similar effects. Later the preliminary announcement[6] of the present work led Murlin and Kramer[7] to try unboiled pancreatic extract. This was given by mouth, however, and was mixed with Na_2CO_3. A favorable influence on the respiratory quotient was observed in two experiments.

The present work was undertaken because of the remarkable influence which an emulsion of pancreas had been found to exert upon the disposition of intravenously injected dextrose in diabetic dogs. In normal animals[8] large quantities of sugar, introduced intravenously, were promptly lost from the circulation; in diabetic animals,[6,9] on the other hand, this occurred very slowly, as was shown by the fact that even an hour and a half after the end of the sugar infusion the blood sugar was still far above its original level. Now, when an emulsion of pancreas was mixed with the glucose solution to be injected, the diabetic animal handled the sugar in nearly a normal manner.[6] Then the question naturally arose whether a similar pancreatic emulsion would not help the diabetic organism to dispose of its own excess sugar also.

In testing this question there were two principles which, we felt, should be followed. First, since the chemical properties of the effective substance or substances in the pancreas were unknown, it seemed necessary to avoid complicated procedures of purification; therefore, a simple water extraction with subsequent dilution with saline was adopted. Secondly, since depancreatization brings on its effect within a very short time, it appeared that normally the pancreas secretes its effective substance into the blood stream continually in very small amounts. It was therefore decided to

[5] Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1913, xv, 365.

[6] Kleiner, I. S., and Meltzer, S. J., *Proc. Nat. Acad. Sc.*, 1915, i, 338.

[7] Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1916, xxvii, 517.

[8] Kleiner, I. S., and Meltzer, S. J., *Am. J. Physiol.*, 1914, xxxiii, p. xvii. Kleiner, I. S., *J. Exp. Med.*, 1916, xxiii, 507.

[9] Kleiner, I. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 58.

introduce the pancreatic preparation by the intravenous route very slowly and over an appreciable period; the results, however, might be expected to be observed during the injection and for only a few hours thereafter. These principles were followed and the results were very gratifying—in a word, the slow intravenous infusion of an aqueous pancreatic "emulsion" usually resulted in a temporary but marked decrease in the glycemia and glycosuria. The first few experiments were briefly reported in 1915.[6] The present paper includes these experiments as well as a large number of experiments performed since the first communication.

Method.

Operation.—Total depancreatization was performed in the majority of cases. In three animals, used in the six most recent experiments, the "Allen" procedure, *i.e.* leaving a small remnant around the large pancreatic duct, was adopted. The operations were performed under ether anesthesia.

Preparation of "Emulsion".—Fresh dog's pancreas was hashed, mixed with three or four times its weight of sterile distilled water, and placed in the refrigerator. After a period of from 1 to 20 hours it was strained and squeezed through muslin. The fluid thus obtained was diluted with 5 volumes of sterile 0.9 per cent NaCl solution before injection. This dilute solution was faintly acid or neutral to litmus, light pink in color, and almost clear. At no stage was it filtered, nor was sodium carbonate or any other substance added. In one experiment (LP77a), Ringer's solution was used in the extraction and also in the dilution; this did not lead to a better result. The emulsions of other tissues, used in the control experiments, were prepared in a similar manner.

Injection and Blood Sampling.—Some time before the infusion was to be given, the animals (in most of the experiments) received a small dose of morphine sulfate subcutaneously, usually about 1.5 mg. per kilo. Cannulas were then introduced under local anesthesia into a convenient vein and artery, for injection and blood sampling, respectively. In the six recent experiments no morphine was given and only one cannula (the venous) was introduced under local anesthesia (ethyl chloride). In this series

the blood was drawn directly from an external jugular vein into a syringe. The urine was obtained by catheter in all cases.

Analytical Methods.—The blood sugar determinations were made by the Myers-Bailey[10] modification of the Lewis-Benedict method. Glucose in the urine was estimated by a modification of the Pavy[11] method or by Benedict's[12] method. Hemoglobin was determined in most of the experiments by discharging 2 cc. of blood into dilute HCl (about 0.3 per cent) in a 250 cc. volumetric flask, diluting to this volume, and comparing the density of the color in a Duboscq colorimeter. The first blood sample of each experiment was used as the standard and was taken as 100 per cent for that experiment. Later the Sahli hemoglobinometer was used and, to avoid confusion, the results are similarly expressed.

Four illustrative protocols, including three injections of pancreas emulsion and one control, follow.

Experiment LP72a.—From Table I it will be observed that the high blood sugar value of 0.31 per cent fell to 0.14 per cent in the course of the 78 minute injection period and after another period of 90 minutes it was still low (0.13 per cent). Both of these figures, 0.14 and 0.13 per cent, are practically normal values, but emphasis is to be placed on the extent of the drop, from 0.31 to 0.13 per cent, a fall of 0.18 per cent, rather than upon the absolute value finally obtained. At the same time this decrease in the concentration of the blood sugar is not accompanied by any change in the hemoglobin value, or by an increased glycosuria. In other words it is not due to a dilution of the blood sugar by the injection fluid, or to a washing out of the sugar through the kidneys. In fact the kidney excretion of sugar is greatly diminished.

This was one of the few experiments in which the injection produced any noticeable general symptoms; there was first a fall and then a rise in temperature. There was no marked harmful effect of the infusion, however, as the animal was given a second infusion 2 days after the first and lived 2 days after that. The second infusion caused only a slight fall in the glycemia (see LP72b in Table V).

[10] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.
[11] To be published shortly.
[12] Benedict, S. R., *J. Biol. Chem.*, 1911, ix, 57.

TABLE I.

Experiment LP72a.

Male, 11.25 Kilos, Completely Depancreatized April 19, 1915.

Date.	Time.		Blood sugar.	Hemoglobin in per cent of first sample.	Urine.			Temperature.
					Volume	Glucose.		
	a.m.		*per cent*		*cc.*	*per cent*	*gm. per hr.*	°C.
Apr. 20	10.00	Injected 1.5 mg. of morphine sulfate per kilo subcutaneously.						
	10.23							39.1
	10.36	Cannulas have been introduced into left external jugular vein and right carotid artery under ethyl chloride and cocaine hydrochloride.						
	11.08				20	10.08		
	11.11		0.31	100				
	11.15–	Injected intravenously						
	12.33	112 cc. of dilute pancreas emulsion prepared from the pancreas of this dog.						
	11.23	Cheyne-Stokes respiration during most of injection.						
	p.m.							
	12.34		0.14	101				37.9
	12.44				31.5	8.79	1.85	
	12.48							38.8
	1.00	Slight tremors of muscles of head and hind legs.						
	1.18							40.3
	1.35	Tremors have stopped.						
	2.04		0.13	100				
	2.15				42.5	1.62	0.46	
	2.18							40.8
	a.m.							
Apr. 21	9.45	Not catheterized.			193	1.49		39.3

Experiment LP76.—In Experiment LP76 (Table II), in spite of an intense hyperglycemia and glycosuria the administration of

TABLE II.

Experiment LP76.

Female, 9 Kilos, Completely Depancreatized May 11, 1915.

Date.	Time.		Blood sugar.	Hemoglobin in per cent of first sample.	Urine.			Temperature.
					Volume.	Glucose.		
	a.m.		*per cent*		*cc.*	*per cent*	*gm. per hr.*	°C.
May 12	10.15							38.5
	10.40	Injected 1.4 mg of morphine sulfate per kilo subcutaneously.						
	11.06	Cannulas have been introduced into left external jugular vein and left carotid artery under ethyl chloride and cocaine hydrochloride.						
	11.07	Catheterized.			111	+++		
	11.20							38.0
	11.43				20.5	11.16	3.82	
	11.45		0.33	100				
	11.49–12.52	Injected intravenously 112 cc. dilute pancreas emulsion prepared from the pancreas of this dog.						
	p.m.							
	12.53				30	12.38	3.18	
	12.54		0.25	101				
	1.13							39.0
	2.27				14	3.64	0.33	
	2.29		0.23	103				
	2.43							39.5

pancreatic emulsion reduced the blood sugar and urine sugar to a marked degree, (0.10 per cent), without producing any unfavorable symptoms or affecting the temperature very much. 2 days

later a different type of experiment was performed on this animal, after which it was killed with chloroform.

Experiment DP1a.—Experiment DP1a (Table III) was one of the more recent ones. No morphine was used and only an in-

TABLE III.

Experiment DP1a.

Female, 8.4 Kilos, 92 Per Cent of the Pancreas Removed on April 7, 1919, the Remnant Left Being in Communication with the Large Duct.

Date.	Time.		Blood sugar.	Hemoglobin in per cent of first sample.	Urine.			Temperature.
					Volume.	Glucose.		
	a.m.		per cent		cc.	per cent	gm. per hr.	°C.
Apr. 8	10.20		0.20	100				
	10.36	Catheterized.				1.25		
	11.19							38.8
	11.30	Under ethyl chloride a cannula has been inserted in the right external jugular vein.						
	11.33		0.21	102				
	11.43				16	8.0	1.15	
	11.49–12.31	Injected intravenously 60 cc. of dilute pancreas emulsion prepared from the pancreas of this dog.						
	p.m.							
	12.36		0.12	102				
	12.44				10	5.40	0.53	
	2.15		0.08	96				
	2.23				14	0.5	0.04	
	2.26							40.4
	a.m.							
Apr. 9	10.08		0.27	99	800	0.33	0.20	
	10.20	Catheterized.			20	3.62		
	10.26							38.5

jection cannula was introduced into a vein. The animal was kept on the board only during the necessary experimental procedures. The infusion of pancreas emulsion in this case caused no unfavorable symptoms and had a very striking effect on the glycemia.

Before injection there was a constant and moderate hyperglycemia (0.20 and 0.21 per cent). At the end of the injection, the blood sugar had fallen to 0.12 per cent, practically a normal figure, and 1 hour and 39 minutes later the distinctly normal value, 0.08 per cent, was found. On the next morning hyperglycemia had again been established. The urinary sugar record is also of interest.

TABLE IV.

Experiment LP81a.

Male, 14.25 Kilos, Depancreatized June 7, 1915, at least 94 Per Cent of the Pancreas Removed.

Date.	Time.		Blood sugar.	Hemoglobin in per cent of first sample.	Urine.			Temperature.
					Volume.	Glucose.		
	a.m.		*per cent*		*cc.*	*per cent*	*gm. per hr.*	*°C.*
June 8	10.35	Injected 1.5 mg. of morphine sulfate per kilo subcutaneously.						
	11.19	Cannulas have been introduced into the left carotid artery and right jugular vein under ethyl chloride.						
	11.24							37.8
	11.43	Catheterized.			116	5.96		
	11.52		0.34	100				
	11.56–	Injected intravenously						
	12.39	114 cc. of dilute emulsion of normal dog's						
	p.m.	submaxillary gland.						
	12.41		0.32	95				
	1.08							38.3
	2.14		0.33	94				
	2.38				100	3.32	1.14	

The excretion of 1.15 gm. of glucose per hour before the injection was followed by a diminution to half that figure during the injection and thereafter only traces were excreted for at least an hour and a half (0.04 gm. per hour). The over night urine also contained very little sugar (0.2 gm. per hour) but it is evident that the kidney was not rendered permanently impermeable to

sugar because the next urine obtained by catheter contained 3.62 per cent dextrose.

On the following day (see DP1b in Table V) an injection of another pancreas preparation lowered the blood sugar from 0.27 to 0.20 per cent. The urinary sugar excretion was diminished very markedly; in one period no qualitative reaction whatever could be obtained.

Experiment LP81a.—In Table IV is given an outline of Experiment LP81a, one of the control experiments. The animal received an intravenous injection of submaxillary gland emulsion. The slight decrease in the blood sugar percentage is in marked contrast with the results obtained when pancreas emulsion was used, and runs parallel with the hemoglobin; *i.e.*, here what little effect there is may be referred to dilution by the intravenous injection of 114 cc. of fluid. The urinary data are insufficient in this experiment but indicate a possibly diminished output of sugar.

There were no noticeable general effects caused by the infusion and *on the next day the administration of pancreatic emulsion lowered the blood sugar from 0.38 to 0.24 per cent* (see LP81b, Table V).

In Table V all the experiments with pancreas emulsions are collected. It will be seen that in most of them there occurred a substantial reduction in the blood sugar. As the samples were not taken at short intervals it is quite probable that the lowest values were not discovered. However, normal or nearly normal figures were found in several cases. Study of the table indicates that, when effective, the emulsion produces an effect during the slow injection, because the blood sugar is usually lower at the end of the injection than at the beginning. The glycemia remains low or continues to fall for about $1\frac{1}{2}$ to $1\frac{3}{4}$ hours, and then, if we may trust the few figures at hand, it gradually rises. It is certain that the effect does not last many hours, for in a number of the experiments a sample of blood was taken on the following morning and invariably showed a hyperglycemic value.

In all except one experiment (LP75), there occurred a diminution in the excretion of sugar. In some cases this was quite marked, resulting in a urine reacting negatively to Benedict's qualitative test. Such urines, however, had normal color and specific gravity. In about half the experiments there was a

TABLE V.

Injection of Pancreatic Emulsions into Depancreatized Dogs.

Experiment No.	Days after operation.	Blood sugar.				Sugar output in urine per hour.					Emulsion prepared from pancreas of same or different animal.	Remarks.
		Before injection.	End of injection.	After injection.		Before injection.	During injection.	After injection.				
		per cent	per cent	min.	per cent	gm.	gm.	hrs.	gm.	cc.		
LP70	2	0.28	0.09	92	0.08		0.92	1.5	Tr.	1.4	Different.	This dog used for other experiments on preceding day and again 5 days later.
LP72a	1	0.31	0.14	91	0.13		1.85	1.5	0.46	42.5	Same.	Slight symptoms produced by injection (see protocol, Table I). Used 2 days later for Experiment LP72b.
LP72b	3	0.28	0.22	93	0.22		0.88	1.4	0.61	15	"	No symptoms.
LP73	2	0.33	0.25	71	0.16		→	0.8	+	5*	"	Dog died 50 min. after end of injection; heart blood obtained 21 min. later. Peritonitis; perhaps 25 per cent of pancreas not removed.
LP74a	1	0.27	0.21	93	0.18		5.07	1.6	0.66	9	"	Used 4 days later for Experiment LP74b.
LP74b	5	0.23	0.18	92	0.13		1.54	1.5	0.07	5.5	Different.	Dog used on the next day for another experiment.
LP75	2	0.27	0.22	101	0.23		1.14	1.8	1.55	34	"	Killed 3 days later; about 8 per cent of pancreas not removed.
LP76	1	0.33	0.25	97	0.23	3.82	3.20	1.6	0.32	14	Same.	Used this dog 2 days later for another experiment.
LP77a	1	0.21	0.17	95	0.16	1.26	0.66	1.6	0.05	23	"	Ringer's solution used in preparing emulsion; pregnant animal; used 6 days later for LP77c.
LP77c	7	0.24	0.25	90	0.22		→	1.5	0.2*	23.5*	Different.	Dog used 6 days before for LP77a, and 4 days before for LP77b (Table VI).

LP81b	2	0.38	0.26	90	0.24		1.79	1.7	0.09	22.5	Different.	Received injection of submaxillary emulsion on preceding day (Experiment LP81a, Table VI); killed 2 days later; perhaps 6 per cent of pancreas not removed.
DP1 a†	1	0.20	0.12	104	0.08	1.16	0.54	1.7	0.04	14	Same.	"Allen" procedure; 8 per cent of pancreas left; used 2 days later for Experiment DP1b.
		0.21						20	0.17	820		
DP1 b†	3	0.25	0.21	94	0.20	>1.15	1.25	2.2	0.00	60	Different.	Killed on following day.
		0.27		240	0.21			1.9	0.08	65		
								18	0.44	260		
DP2 a†	2	0.31	0.24	107	0.19	1.00	0.88	1.7	0.04	38	Same.	"Allen" procedure; 4 per cent of pancreas left; used 3 days later for Experiment DP2b.
		0.30						18	0.00	425		
DP2 b†	5	0.30	0.26	81	0.17	1.20	1.34	1.1	0.00	26	Different.	Had been used 3 days before for Experiment DP2a; used 3 days later for Experiment DP2c.
		0.33‡		164	0.24			1.4	0.42	83		
								18.5	0.55	583		
DP2 c†	8	0.33	0.29	140	0.26	1.85	0.26	2.0	0.00	39	Same.	Had been used for Experiments DP2 a and b.
				246	0.30	(24 hrs.)		1.9	0.25	66		

* During and after injection.
† No morphine given.
‡ After feeding.

TABLE VI.

Injections of Emulsions of Tissues, Other than Pancreatic, into Depancreatized Dogs.

Experiment No.	Days after operation.	Blood sugar.				Sugar output in urine per hour.					Tissue used for emulsion.	Remarks.
		Before injection.	End of injection.	After injection.		Before injection.	During injection.	After injection.				
		per cent	*per cent*	*min.*	*per cent*	*gm.*	*gm.*	*hrs.*	*gm.*	*cc.*		
LP77 b	3	0.23	0.26	92	0.28	1.07	→	1.8	0.15*	23.5*	Submaxillary gland.	Had been used 2 days before for Experiment LP77a; used 4 days later for Experiment LP77c (see Table V); pregnant.
LP81 a	1	0.34	0.32	95	0.33		→	2.0	1.14*	100*	" "	Used on the following day for Experiment LP81b (Table V); perhaps 6 per cent of pancreas not removed.
LP84	1	0.31	0.31	92	0.36		→	1.8	0.08*	2.2*	" "	Required 4.2 mg. of morphine per kilo; considerable depression; died during night; cause of death could not be found at autopsy; 4 per cent of pancreas not removed.
LP86	1	0.39	0.37	92	0.42	1.9	→	1.8	0.27*	11*	" "	Killed on following day.
LP83	1	0.41	0.36	101	0.35		4.75	1.7	2.12	28	Spleen.	Died 3 days later of pulmonary edema.
DP3†	5	0.31	0.32	114	0.32		0.28	3.0	0.20	26	Voluntary muscle.	"Allen" type; at time of this experiment glycosuria was not marked; had been used 4 days before for another experiment; chloroformed 6 weeks later; no morphine used.
				193	0.32			21.5	>1.9	830		

*During and after injection.

†No morphine given.

decided reduction in the volume of urine secreted in the 1½ hour period following the injection, indicating perhaps some temporary functional damage to the kidney; it is possible that this may be caused by the injection of any tissue extract. In the rest of the experiments there was no diminution in urinary flow even when the sugar output had practically ceased (see Experiments LP77a, DP1 a and b, DP2 a, b, and c, Table V).

The controls in which emulsions of several other tissues were injected are tabulated in Table VI. There was no marked effect

TABLE VII.

Summary.

Maximum Reduction of Blood Sugar Produced by Injection of Tissue Emulsion.

Marked reduction.			Moderate or no reduction.		
Experiment No.	Maximum reduction of blood sugar.	Kind of emulsion.	Experiment No.	Maximum reduction of blood sugar.	Kind of emulsion.
LP70	0.20	Pancreas.	LP72b	0.06	Pancreas.
LP72a	0.18	"	LP75	0.05	"
LP73	0.17	"	LP77a	0.05	"
LP74a	0.09	"	LP77c	0.02	"
LP74b	0.10	"	DP1b	0.07	"
LP76	0.10	"	DP2c	0.07	"
LP81b	0.14	"	LP77b	Increase.	Submaxillary gland.
DP1a	0.13	"	LP81a	0.02	" "
DP2a	0.11	"	LP84	Increase.	" "
DP2b	0.16	"	LP86	0.02	" "
			LP83	0.06	Spleen.
			DP3	Increase.	Muscle.

on the blood sugar in any of these tests. Injection of emulsion of spleen (Experiment LP83) was followed by a slight fall of blood sugar, from 0.41 to 0.35 per cent; this was the maximum effect observed in any of the controls. Indeed a slight increase in the blood sugar was observed in three experiments. The amount of sugar eliminated by the kidney was usually decreased to some extent, but not so much as in the pancreas injection experiments. In none of these controls, for instance, was a negative or weak Benedict's test obtained and it is probable that in some of these, as in many of the pancreas emulsion experi-

ments, we have to do with an impairment of the renal epithelium. The striking point in these six experiments is the regular failure to observe a marked lowering of the blood sugar as contrasted with the success when pancreas emulsion was used.

This difference is brought out in the summary (Table VII). Here it will be observed that ten out of sixteen pancreas emulsion injections resulted in a marked reduction of the blood sugar, the greatest reduction in each case falling in the range 0.09 to 0.20 per cent. The other six pancreas experiments, as well as all the controls, showed a reduction of only 0.07 per cent or less. Only one of all the sixteen pancreas experiments had practically a negative result (LP77c), while only one of the controls showed anything resembling a positive effect (LP83).

DISCUSSION.

Many investigators[13] have recognized that the best evidence for the internal secretion theory of the origin of diabetes would be an antidiabetic effect of a pancreatic preparation, administered parenterally. The experiments just described show that such a result has been obtained, since the reduction of hyperglycemia is surely an antidiabetic effect, and the reduction of glycosuria is possibly partly of the same nature. The demonstration of the actual combustion of sugar has not been attempted although the additional proof, while not absolutely essential, would be welcome. A favorable effect upon the glycemia and glycosuria has, however, been established and there remain only a few points to be considered.

The reduction in glycemia is real, that is, it is not due to dilution. The hemoglobin values, given in the protocols, indicate that this is the case; the same holds true for all the pancreas injection experiments, although this has not been recorded in Table V. Furthermore, that it is not an accident, a mere coincidence, is proven by the fact that it occurred to a marked degree in ten out of sixteen experiments, and to some extent in fifteen out of sixteen. The controls include only one with any effect—and that an insignificant one—on blood sugar. In some cases

[13] See, for example, Hédon, E., Travaux de Physiologie, Paris, 1898, 133. Pflüger, E., *Arch. ges. Physiol.*, 1907, cxviii, 271.

the pancreas emulsion injection resulted in changing a pronounced hyperglycemia to a normal blood sugar. It is quite likely that more of these cases occurred than were discovered, because the blood samples were not taken at short intervals.

No increased glycosuria occurred which could be said to account for the decrease in blood sugar. On the contrary, the elimination of sugar was decreased in every experiment except one, this being one in which the effect on the blood sugar was slight (Experiment LP75, Table V). The effect upon the urinary sugar generally lasted longer than that upon the blood sugar, but specimens of urine obtained by catheterization on the day following the injection usually contained high percentages of sugar. This would indicate that if the injection of an organ extract decreases the permeability of the kidney to sugar by a toxic action, the effect is only temporary. Since in the controls a similar, but less marked, transitory reduction of the urinary sugar output was observed, it is evident that *the mere reduction of glycosuria is no proof of a beneficial effect of any agent*. Observations of other investigators may be cited in this connection. Murlin and Kramer[5] in one experiment found that an intravenous injection of a mixed boiled extract of pancreas and duodenal mucosa produced a marked fall in the sugar output of a diabetic dog, but that there was a *rise* in the blood sugar. Hédon[14] has shown that transfusion of normal blood into diabetic animals may result in a diminished output of sugar with little if any reduction of the blood sugar level. He assumes that the diabetic complex consists of two phases, of which the renal is the first to be affected by the internal secretion of the pancreas. Is it not possible, however, that the introduction of a foreign blood had a toxic effect on the kidney? In fact Hédon himself admits that cross circulation between two diabetic dogs also diminishes glycosuria. The same criticism applies to all blood transfusion and related investigations[15, 16] in

[14] Hédon, E., *Compt. rend. Soc. biol.*, 1911, lxxi, 124.

[15] Forschbach, J., *Arch. exp. Path. u. Pharmacol.*, 1908-09, lx, 131. This author united animals of the same litter (parabiosis) and later removed the pancreas of one, without causing more than a mild glycosuria. In one experiment (No. 5, p. 145) he reports a blood sugar of 0.32 per cent while the urine was sugar-free.

[16] Drennan, F. M., *Am. J. Physiol.*, 1911, xxviii, 396.

which only the urine was examined. The same question might be asked regarding Scott's work,[4] for he, too, used only the urinary output as an index of the action of his pancreatic extract.

It must be emphasized that the beneficial effects of the pancreas emulsion are not to be referred to sodium carbonate or any other alkali. *No alkali was used in preparing the pancreas emulsions.* The extractions were made with water and the dilutions of the extract, or emulsion, with 0.9 per cent NaCl. The resulting injection fluid was faintly acid to litmus. Our experiments, therefore, cannot be compared with Murlin and Kramer's [5,7] because they always used an alkaline medium. The alkalinity itself is, according to these authors, largely responsible for the beneficial action of the various tissue extracts which they administered. In our experiments, on the other hand, the favorable effect of the pancreatic emulsion must be due to some ingredient peculiar to it, because no alkali was used in its preparation, and because the other tissue emulsions, prepared in a similar manner, had no such favorable effects.

The mechanism of the effect on the blood sugar may be considered briefly. There are several possibilities, corresponding to the several hypotheses for the mechanism of diabetes. The author does not wish to enter here into an extended discussion of these hypotheses, but wishes to point out how the results of this investigation apply in each case. For the "overproduction" theory the injection fluid may be held to contain a substance which decreases the output of sugar by the liver. If one believes that, in diabetes, the cells themselves lack the power to burn sugar—as many investigators do—it will be said that the pancreas emulsion contains the substance which stimulates the cells to this activity, or the "amboceptor" (Allen) which enables the cells to anchor sugar to their protoplasm.

Kleiner and Meltzer[17] have indicated another explanation for some of the phenomena of diabetes; *i.e.*, a decreased permeability to sugar of the capillary endothelia and perhaps of other cells as well. Palmer[18] has corroborated this by showing that the striated muscle of diabetic animals has a lower concentration of glucose

[17] Kleiner, I. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 58.

[18] Palmer, W. W., *J. Biol. Chem.*, 1917, xxx, 79.

than that of normal animals, if the respective levels of blood sugar are taken into account. We have also reported that pancreas emulsions enable diabetic animals to handle, in a nearly normal manner, large amounts of intravenously administered dextrose. This was taken to mean that the pancreas emulsion had restored the permeability, thus permitting the sugar to be absorbed and taken care of in the normal manner. The same explanation may be applied to the present experiments. In other words the pancreas emulsion perhaps contains some substance which alters the permeability of the capillary walls, allowing the diabetic sugar to go through them into the tissues. Another factor which may come into consideration is the state of the blood sugar. The author has presented evidence[19] that the diabetic blood sugar is in a combined or poorly diffusible state. This indicates a further difficulty presented to the diabetic organism in its struggle to obtain sugar for its starving tissues. The enzymes present in the pancreas emulsion may be able to break up this combination setting free crystalloid glucose. This can diffuse readily through the tissues which at the same time have been rendered more permeable to it. This last possibility may be tested directly by the method already described.[19]

The question of the general toxicity of the extracts used requires a few words. It might be expected that an unfiltered tissue extract of this sort would produce grave symptoms when injected intravenously. This was not the case, however. The explanation seems to be that the extract was highly diluted and very slowly injected. In but few instances among the entire list of experiments was any depression or other unfavorable symptom observed. Furthermore, most of the animals received several infusions on different days and survived them. One dog died shortly after a pancreas emulsion injection, but death was apparently due to peritonitis. One control died a few hours after the injection of submaxillary emulsion, and another died of pulmonary edema 3 days after spleen emulsion had been administered.

The fact that these pancreas emulsions lower blood sugar in experimental diabetes without causing marked toxic effects indicates a possible therapeutic application to human beings. It is

[19] Kleiner, I. S., *J. Biol. Chem.*, 1918, xxxiv, 471.

true that the numerous attempts at pancreas therapy have been almost uniformly unsuccessful. However, the methods heretofore used have not resembled the one used in our experiments. This method is based on certain principles, discussed early in this paper, and is extremely simple. It is possible that the temporary effect which it produced in dogs might be duplicated in man and might be useful in certain emergencies. However, it is doubtful whether attempts along this line should be made until further knowledge has been obtained. It is important to know whether a filtered extract would be effective; and particularly whether an emulsion of the pancreas of another species would have its effect when injected into a diabetic dog. Other questions also arise. Do such injections raise the respiratory quotient? Could other paths of injection and other animals be used equally well? Finally the search for the effective agent or agents, their purification, concentration, and identification are suggested as promising fields for further work.

SUMMARY.

Diabetic dogs were given intravenous injections of unfiltered water extracts of fresh pancreas, diluted with 0.9 per cent NaCl solution. The preparation was administered very slowly and usually resulted in a marked decrease in the blood sugar. There was no compensating increase in urinary sugar, but rather a decrease, which may be partly due to a temporary toxic renal effect.

The result is regarded as further evidence for the internal secretion theory of experimental diabetes.

CRYSTALLINE GUANYLIC ACID.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 30, 1919.)

The theory of the polynucleotide structure of the yeast nucleic acid has been receiving confirmation in recent years through the investigations of several workers. Levene,[1] Jones and Kennedy,[2] and Thannhauser and Dorfmüller[3] have described crystalline mononucleotides obtained from yeast nucleic acid. The present communication contains a report on guanylic acid obtained in crystalline form. It crystallized in the form of long prismatic needles having the same appearance as guanosine. The substance had all the properties of guanylic acid. It gelatinized in the presence of mineral impurities. A test for the presence of free phosphoric acid was negative. It had no melting point but turned brown at 208°C. The optical rotation in water solution was $[\alpha]_D^{20} = -7.5$, and in a 5 per cent ammoniacal solution $[\alpha]_D^{20} = -43.5$. On hydrolysis it gave guanosine or guanine sulfate depending on the conditions of the experiment. The free acid, when air-dry, crystallized with 2 mols of crystal water, and, when dried under diminished pressure at the temperature of a toluene vapor bath to constant weight, still retained $\frac{1}{2}$ mol of crystal water. The pure guanylic acid was converted into a crystalline brucine salt.

EXPERIMENTAL.

Crude brucine salt of guanylic acid was converted into the ammonium salt in the manner described in a previous publication.[4] The ammonium salt was dissolved in hot water. The resulting

[1] Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 21.

[2] Jones W., and Kennedy, R. P., *J. Pharmacol. and Exp. Therap.*, 1919, xii, 253.

[3] Thannhauser, S. J., and Dorfmüller, G., *Z. physiol. Chem.*, 1919, civ, 65.

[4] Levene, P. A., *J. Biol. Chem.*, 1919, xxxix, 77.

solution was acidulated, and the substance precipitated by means of neutral lead acetate. The precipitate was repeatedly washed with water, then suspended in water, and freed from lead. The resulting solution was again precipitated with neutral lead, and the process repeated once more. Finally the solution obtained on decomposition of the lead salt gave on concentration under diminished pressure a crystalline deposit.

On drying under diminished pressure at the temperature of xylene vapor, the substance still retained $\frac{1}{2}$ mol of crystal water.

0.1084 gm. of the vacuum-dry substance gave 0.1274 gm. of CO_2 and 0.0424 gm. of H_2O.

0.1834 gm. of the substance used for Kjeldahl nitrogen estimation required 24.45 cc. of 0.1N acid for neutralization.

0.2757 gm. of the substance gave 0.0844 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5O_8+\frac{1}{2}H_2O$.	Found.
	per cent	*per cent*
C	32.24	32.05
H	4.03	4.38
N	18.80	18.67
P	8.33	8.55

In 10 per cent hydrochloric acid the substance was optically inactive. In aqueous solution the rotation was

$$[\alpha]_D^{20} = \frac{-0.15 \times 100}{1 \times 2} = -7.5$$

In 5 per cent aqueous ammonia solution the rotation was

$$[\alpha]_D^{20} = \frac{-0.87 \times 100}{1 \times 2} = -43.5$$

Acid Hydrolysis of the Substance.

2.0 gm. of the substance were dissolved in 20.0 cc. of 2 per cent sulfuric acid and boiled over a flame with a return condenser for 1 hour. The substance was filtered and allowed to cool. Soon a crystalline deposit formed. This was filtered and recrystallized out of 5 per cent sulfuric acid. The substance was dried over night over sulfuric acid in a vacuum desiccator.

0.0905 gm. of dry substance gave 27.6 cc. of nitrogen gas at $T° = 25°$ C and $P = 763$.

	Calculated for $(C_5H_5N_5O)_2H_2SO_4$.	Found.
	per cent	*per cent*
N	35.00	35.08

Ammonia Hydrolysis.

2.5 gm. of the substance were dissolved in 50 cc. of water containing 5 cc. of ammonia water, and the solution was made up to 75 cc. It was then heated in a sealed tube for 4 hours at 135°C. On cooling, the contents of the tube practically solidified. The solid material was filtered on suction and the residue recrystallized twice out of water. The crystals had the characteristic appearance of guanosine. The yield of the recrystallized substance was 0.6 gm.

0.100 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.8 cc. of 0.1N acid.

	Calculated for $C_{10}H_{13}N_5O_5 \cdot 2H_2O$.	Found.
	per cent	*per cent*
N	21.94	21.98

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.66 \times 100}{1 \times 1} = -66.0$$

Since this rotation was slightly higher than the one previously recorded (−60.5), and since in the earlier work a less sensitive instrument was employed for the measurement, the rotation of a pure sample of guanosine was measured under exactly the same conditions as the sample obtained in the present work. The value obtained was

$$[\alpha]_D^{20} = \frac{-0.66 \times 100}{1 \times 1} = -66.0$$

Brucine Salt.

2 gm. of the crystalline guanylic acid were dissolved in water and neutralized by means of a solution of brucine in methyl alcohol. A crystalline deposit formed immediately. This was

filtered off and recrystallized out of a 35 per cent solution of alcohol in water. The air-dry substance contracted at 217° (Anschutz thermometer), melted into a brown liquid at 233°, and effervesced at 240°C.

0.1000 gm. of the substance gave on combustion 0.1938 gm. of CO_2 and 0.0558 gm. of H_2O.

0.2000 gm. of the substance gave 18.2 cc. of nitrogen gas at $T° = 25°$ and $P = 759$.

0.300 gm. of the substance gave 0.262 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{20}H_{34}N_9O_{18}P_3 \cdot 7H_2O$.	Found.
	per cent	*per cent*
C	52.61	52.85
H	6.78	6.24
N	9.88	10.41
P	2.43	2.43

The rotation of the substance in 35 per cent alcohol solution was

$$[\alpha]_D^{20} = \frac{-0.52 \times 100}{1 \times 2} = -26.0$$

A NEW STEROL.

By TAKEO IKEGUCHI.

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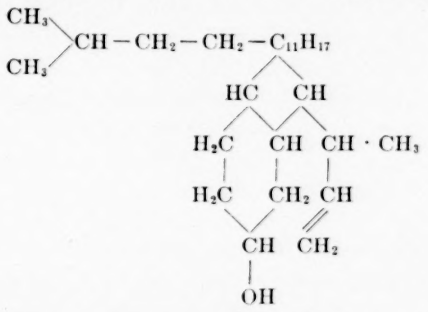
(Received for publication, September 17, 1919.)

The sterols are probably contained in all cells, and there seems to be no doubt of their importance physiologically. Until now, however, the only property which has been conclusively proved is their antidotal effect toward the hemolytic action of the saponins.

If we take into account the poisonous action of some of the sterol derivatives upon the tissues of the heart, the biological significance of sterol affords an interesting field for research.

Whereas cholesterol, which is commonly found in animals, has received much attention, and its structure has been practically confirmed by Mauthner, Abderhalden, and especially by Windaus,[1]



the other members of the sterol group, which occur in plants and fungi, have received but slight consideration.

Several varieties of sterol have been isolated from fungi. Tanret[2] obtained ergosterin and fungisterin from ergot; and Zellner[3] separated a sterol, with the formula $C_{26}H_{40}O$, from the

[1] Windaus, A., *Ber. chem. Ges.*, 1912, xlv, 2421.

[2] Tanret, quoted by Windaus, A., in Abderhalden, E., Biochemisches Handlexikon, Berlin, 1911, iii, 309.

[3] Zellner, J., quoted by Fodor-Halle, A., in Abderhalden, E., Biochemisches Handlexikon, Berlin, 1914, viii, 493.

toadstool. Up to the present, however, no one appears to have separated one identical sterol from different species of fungi, comparing it with other sterols and determining its structure. This is the reason for attempting investigation along this line.

In an earlier treatise,[4] the writer described a sterol-like substance which he obtained from the *Lycoperdon*. During the course of the present research, he examined *Collybia shiitake*, from which he isolated another sterol in pure form. This he traced through *Armillaria edodes*, *Hydnum asparatum*, and *Lycoperdon gemmatum*.

Method of Preparation and General Tests.

100 gm. portions of each of the above mentioned fungi, dried as well as possible at room temperature, and then thoroughly in an oven at 70°C., were extracted with ether. The ether was then distilled, leaving in each case an oily residue, which soon coagulated into a cheesy mass containing the sought for crystals of sterol. The crystals were washed with petroleum ether and alcohol, and then recrystallized from a hot solution of the latter solvent. Each batch of fungi, treated in this manner, yielded about 0.1 gm. of long, colorless, hexagonal crystals.

All the crystals obtained gave the same color reactions. (1) The crystals were dissolved in chloroform; an equal volume of concentrated sulfuric acid was then carefully added so as to form a separate layer. From the contact surface between the two layers the lower sulfuric acid layer gradually became blood-red, while the chloroform layer turned brownish blue, there being a distinct green fluorescence in both layers. (2) To a chloroform solution of the crystals a little acetic anhydride and two drops of concentrated sulfuric acid were added. The mixture became at first rose-red, then successively blue, and dark green. (3) The crystals were dissolved in acetic anhydride. On adding a few drops of concentrated sulfuric acid the mixture turned first rose-red, then violet, then blue, and finally green. (4) The crystals do not give the oxycholesterol reaction.

Each of the above reactions is similar to the corresponding one for cholesterol. In the first test, however, where cholesterol is

[4] Ikeguchi, T., *Z. physiol. Chem.*, 1914, xcii, 257.

concerned, the coloration shown by the two layers is reversed. It resembles the cholestanon[5] reaction, but can be differentiated from the latter by the difference of color of the chloroform layer.

The crystals obtained, as indicated above, by the extraction of the various fungi are colorless and odorless and melt at 159–160°C. When exposed to the air for a long time or heated above 80°C., they become yellow in color. On ignition they emit an odor resembling that of isovaleric acid. They are readily soluble in ether, chloroform, and warm alcohol, with difficulty in cold alcohol and petroleum ether, and are insoluble in water.

In solution the substance is levorotatory. The sample to be determined was dissolved in chloroform and the measurements were made with a Landolt polariscope, using a 2 dm. tube. The specific rotation of the substances obtained from each fungus was nearly the same

Name of fungus.	Specific rotation $([\alpha]_D^{20})$.
Collybia shiitake	−129.4
Armillaria edodes	−129.23
Hydnum asparatum	−129.55

and the results obtained by analysis were also nearly equal.

Name of fungus.	Weight of sample. gm.	CO_2 gm.	H_2O gm.
Collybia shiitake	0.1464	0.4402	0.1464
Armillaria edodes	0.1086	0.3260	0.1110
Hydnum asparatum	0.1568	0.4746	0.1556

Found.		Calculated ($C_{30}H_{50}O_2$).	
C per cent	H per cent	C per cent	H per cent
82.004	10.81	81.81	10.9
81.87	11.35		
82.02	10.95		

The yield of the substance obtained from the *Lycoperdon* was so small, owing to the difficulty of isolating it from other materials present, it could not be analyzed; however, there is no doubt that it is identical with others, agreeing with them in its crystal form, color reactions, and melting point.

[5] Diels, O., and Abderhalden, E., *Ber. chem. Ges.*, 1904, xxxvii, 3099.

The sterol obtained probably occurs not only in the fungi above mentioned but throughout all classes of fungi, wherefore I shall name the compound mycosterol.

According to the analytical results, mycosterol may be regarded as an oxidation product of stigmasterol,[6] and this, referring to the fact ascertained by Lifschutz that cholesterol is converted into oxycholesterol in the animal body, gives mycosterol an added interest.

In order to ascertain whether mycosterol is capable of preventing the hemolytic action of saponin, an experiment was carried out following Hausmann's method.[7] 5 cc. of an ether solution of mycosterol were gradually added with stirring to 5 cc. of saponin solution (0.1 gm. of saponin in 100 cc. of isotonic sodium chloride solution). The mixture was placed in an oven at 40°C. for 7 to 8 hours, then at 30°C. for 1 to 2 hours, after which the ether evaporated *in vacuo*. 1 and 2 cc. of the mixture thus prepared were added respectively to two 5 cc. portions of a floating solution of red corpuscles taken from a rabbit. The phenomenon of hemolysis occurred to a small extent in 24 hours, while a similar blood solution to which the saponin solution only had been added showed complete hemolysis instantaneously. Another similar solution with cholesterol in place of the mycosterol showed no hemolysis at all even after 24 hours.

This experiment shows that mycosterol, as well as cholesterol, is an antidote against saponin though its power is not so great.

Digitonin Compound.[8]—Mycosterol was dissolved in boiling alcohol, and a 1 per cent alcohol solution of digitonin was added in excess until the mixture presented a slight turbidity. After standing for about 1 hour, crystals separated out which were filtered off, washed with alcohol and ether, and then recrystallized from methyl alcohol after adding a little water. The crystals melted at 242°C. with the evolution of gas.

The crystals are soluble in pyridine, and with difficulty in acetone and benzene. They give the typical Liebermann-Burhard reaction which serves to show that the crystals are a compound of mycosterol and digitonin.

[6] Windaus, A., and Hauth, A., *Ber. chem. Ges.*, 1906, xxxix, 4378.
[7] Hausmann, W., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 567.
[8] Windaus, A., *Ber. chem. Ges.*, 1909, xlii, 244.

0.1320 gm. of substance (dried at 110°C.) gave 0.2998 gm. of CO_2 and 0.1034 gm. of H_2O.

Found.		Calculated ($C_{36}H_{62}O_{30}$).	
C	H	C	H
per cent	*per cent*	*per cent*	*per cent*
61.94	8.70	62.119	8.64

According to Hausmann and Abderhalden, a sterol, containing an hydroxyl group and a double-bond in its molecule, is capable of preventing hemolysis by saponin, and when the hydroxyl group is replaced by another atom or group, or linked with such an atom or group, as is the case in an ether, its action disappears. If the double-bond is saturated, its action becomes weaker than that of the initial material. Referring to this conclusion, mycosterol probably contains a free hydroxyl group, but does not contain a double-bond. To test these assumptions the following experiments were carried out.

Acetylation.—In a small flask fitted with a condenser, 0.5 gm. of mycosterol and twenty times its volume of acetic anhydride were boiled gently on a sand bath for 5 hours. After standing, the crystals which precipitated out were filtered off, washed with acetic acid, and recrystallized from ether, melting at 169°C.

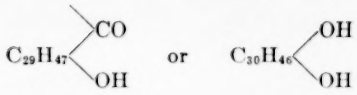
0.1524 gm. of substance gave 0.4448 gm. of CO_2 and 0.1504 gm. of H_2O.

Found.		Calculated ($C_{32}H_{50}O_2$)	
C	H	C	H
per cent	*per cent*	*per cent*	*per cent*
79.59	10.96	79.66	10.37

Bromination.—0.123 gm. of mycosterol was dissolved in 5 cc. of ether and to this solution a bromacetic acid mixture was added. The ether was then allowed to evaporate spontaneously, leaving colorless needle-shaped crystals. The melting point, color reaction, and solubilities agreed with those of mycosterol; therefore, the crystals may be considered to be the unchanged mycosterol.

It is evident from the above experiments that mycosterol contains at least one free hydroxyl group in its molecule, but does not contain a double-bond which can be tested by the methods above mentioned.

With regard to the manner in which the other atom of oxygen is linked up in the molecule of mycosterol, there are several possibilities. Reasoning from the oxidation products of cholesterol, however, the carbonyl group or hydroxyl group



is most probably to be taken into account. Since mycosterol, when treated with phenylhydrazine, does not form a phenylhydrazone, it may be concluded that it does not contain the carbonyl group for which this is a test. If the oxygen may exist as a carbonyl group it should be in a state which cannot be readily acted upon by phenylhydrazine, as oxycholesterol, obtained by Mauthner[9] oxidizing cholesterol, does not readily form a phenylhydrazone, though containing the carbonyl group.

0.5 gm. of phenylhydrazine hydrochloride and 0.5 gm. of sodium acetate were added to 1 gm. of mycosterol dissolved in warm alcohol. The mixture was heated for 1 hour on a water bath under a reflux condenser. Upon cooling crystals precipitated, which were filtered off by means of suction, washed with alcohol, and recrystallized from warm alcohol. The crystal form and the melting point were in agreement with those of mycosterol, and as the compound contains no nitrogen, it must be mycosterol itself.

Oxidation of Mycosterol.—As has already been shown, mycosterol has one hydroxyl group, which is verified by the action of acetic anhydride. Therefore, if the other oxygen exists as a hydroxyl group, it must be in a form which cannot be detected by acetylation, as is often the case with a tertiary alcohol group.[10] The decision between the two possibilities can be made by examining the oxidation products of mycosterol. On treatment of mycosterol with chromic acid it yielded three different neutral crystalline products and an analysis of the substance of which the largest amount was obtained agreed with the compound $C_{30}H_{48}O_3$.

5 gm. of chromic acid dissolved in 10 cc. of acetic acid were carefully added to 10 gm. of mycosterol which were suspended in 100 cc. of acetic acid. The mixture was heated on a water bath until it was deep green. The resultant solution was poured into a diluted sodium chloride solution, the whole then extracted with ether. The ether layer was treated with sodium chloride solution, then with sodium hydroxide solution to remove acid substances.

[9] Mauthner, J., and Suida, W., *Monatsh. Chem.*, 1896, xvii, 582.

[10] Diels, O., and Abderhalden, E., *Ber. chem. Ges.*, 1903, xxxvi, 3178.

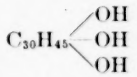
The oily residue mixed with crystals formed on evaporation was washed with petroleum ether and benzene and recrystallized from acetone, melting at 188–189°C.

This substance is soluble in acetone, alcohol, and chloroform, and insoluble in water.

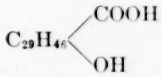
0.118 gm. of substance (dried at 105°C.) gave 0.3404 gm. of CO_2 and 0.1168 gm. of H_2O.

Found.		Calculated ($C_{30}H_{48}O_3$).	
C	H	C	H
per cent	*per cent*	*per cent*	*per cent*
78.67	10.99	78.94	10.53

According to these results the oxidation product may be regarded as an additional product of one atom of oxygen and one molecule of mycosterol, for the number of carbon and hydrogen atoms remains equal to that in mycosterol. Considering the relation of the new oxygen toward the molecule, two possibilities may be suggested; namely, either that one of the groups containing oxygen in the molecule of mycosterol has been converted into a carbonyl group by the addition of one oxygen, or that a new group containing oxygen has been formed.



(1)



(2)

Since the oxidation products are neutral, the former case is out of the question, and since the number of hydrogen atoms in the molecule of the new substance is equal to that of the original mycosterol, the hydroxyl group and the hydrocarbon group must have remained untouched.

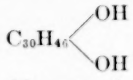
It is, therefore, reasonable to conclude that in the molecule of the oxidation product two out of three groups containing oxygen remain the same as in mycosterol, while the third is a newly formed hydroxyl group. To prove this point 1 gm. of the oxidation product was mixed with twenty times its volume of acetic anhydride and boiled gently on a sand bath for 5 hours. Upon cooling, water was added until the solution became a little turbid. Long needle-shaped crystals were precipitated which were washed thoroughly with a little ligroin and recrystallized from acetic acid, melting point 201–202°C.

0.1647 gm. of substance (dried at 105°C.) gave 0.4454 gm. of CO_2 and 0.141 gm. of H_2O.

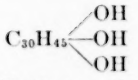
Found.		Calculated ($C_{30}H_{48}O_6$).	
C *per cent*	H *per cent*	C *per cent*	H *per cent*
73.92	9.3	74.22	9.27

The substance which was obtained by treating the oxidation product with acetic anhydride is a triacetic ester as is shown in formula (1).

From this fact it may be concluded that two atoms of oxygen in mycosterol are contained as hydroxyl groups and mycosterol may be a dihydroxyl compound and its oxidation product a trihydroxyl compound.



Mycosterol.



Oxidation product.

ON THE RELATIVE ACCURACY OF COLORIMETRIC AND TITRIMETRIC PROCEDURES FOR DETERMINING NITROGEN AS AMMONIA.

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INTRODUCTION.

The problems arising in connection with investigations in the field of soil biology frequently are concerned with the fixation and transformation of nitrogen and require nitrogen determinations made with greater accuracy than is the case in most other lines of work. While engaged in this field of research, we carried out a considerable number of studies on nitrogen methods. Among other points studied, the comparative accuracy of the colorimetric and titrimetric procedures for the determination of nitrogen in the ammonium form have been investigated.

In the matter of improvement and adaptation of nitrogen methods to particular fields of research, two workers, Folin in America and Mitscherlich in Germany, have been particularly active. Folin, working in the field of animal nutrition, has employed both colorimetric and titrimetric procedures and favors the former, whereas Mitscherlich, studying the problems of plant nutrition, has adhered to the titrimetric procedure. Folin and Farmer[1] used a modified Nessler-Winkler reagent in such a way that as much as 1.5 mg. of nitrogen may be determined colorimetrically without precipitation. By proper aliquoting, the quantity of nitrogen is kept below this amount.

Methods of this type have found quite wide favor, and certain modifications have been proposed from time to time by Folin and

[1] Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.

his associates. On the other hand Mitscherlich and Herz[2] used titration with ± 0.02 N solutions with a surprising degree of accuracy for amounts of nitrogen varying from 0.68 to 6.84 mg. However, it was not clear from the papers of these two workers which procedure was the more accurate, and therefore the work reported below on the probable error of the two procedures, as computed by the method of least squares, was carried out.

EXPERIMENTAL.

Reagents and Solutions.

Indicator Used.—The compound dimethylaminoazobenzene-*o*-carboxylic acid, or methyl red, is the most suitable indicator for the titration of weak bases as ammonia, and is extensively used at the present time. It has rightly supplanted the use of alizarine red, Congo red, cochineal, and laemoid. Its end-point is so sharp, even in the presence of ammonia, that if proper conditions are observed very dilute solutions are titrated with ease and accuracy.

Titrimetric Standards.—Standard 0.02 N acid was prepared from pure sulfuric acid and carbon dioxide-free water, and standardized by the sodium carbonate method, which, according to Mitscherlich and Meeres,[3] is the most accurate. Standard 0.02 N alkali was prepared from sodium hydroxide and carbon dioxide-free water.[4] The titrations were made with the aid of 25 cc. burettes which were of regular 50 cc. length, but were correspondingly smaller in internal diameter, and were graduated to 0.05 cc.[5] These burettes were calibrated by the Bureau of Standards for 20°C., and that temperature was maintained as nearly as possible.

Analytical Standards.—Standard solutions of ammonia were carefully prepared from ammonium hydroxide and from ammonium sulfate. The former were prepared from concentrated C. P. ammonium hydroxide and neutral[6] distilled water, and the

[2] Mitscherlich, E. A., and Herz, P., *Landw. Jahrb.*, 1909, xxxviii, 279.

[3] Mitscherlich, E. A., and Meeres, E., *Landw. Jahrb.*, 1910, xxxix, 345.

[4] We have subsequently found that stock distilled water redistilled over acid permanganate is sufficiently pure for the preparation of standard solutions.

[5] These were obtained from Emil Greiner and Company, New York.

[6] The term "neutral water" as used in this paper refers to water neutral to methyl red.

exact strength determined by a series of titrations against the titrimetric standards. The ammonium sulfate standards were prepared from ammonium sulfate, repurified according to the directions of Folin.

Nessler Reagent.—This reagent was prepared according to the directions of the Standard Methods of the American Public Health Association.[7]

Nessler-Winkler Reagent.—This reagent was prepared according to the original direction of Winkler[8] and used according to Folin and Farmer,[9] who made the observation that if the reagent is diluted just before use (5 cc. to 25 cc.) larger amounts of ammonia may be Nesslerized without precipitation.

Colorimeter.—A study of the Krüss polarization, and Schreiner, Wolff, and Dubosecq colorimeters led to the adoption of the last named as being the most suitable for colorimetric work. The instrument used was of the standard French pattern.

Probable Error of Nesslerization.

In Nesslerization the error lies principally in the difference in the amount of color produced by the action of the reagent on the ammonia and in matching the unknown against the standard.

[7] Methods of water analysis of the American Public Health Association, Standard methods for the examination of water and sewage, Boston, 3rd edition, 1917, 16. 50 gm. of potassium iodide in a minimum quantity of cold water were treated with saturated solution of mercuric iodide until a slight precipitate persists permanently. Add 400 cc. of 50 per cent potassium hydroxide, clarified by sedimentation, and dilute to 1 liter, allow to settle, and decant.

[8] Winkler, L. W., *Chem. Z.*, 1899, xxiii, 541. Hawk, P. B., Practical physiological chemistry, Philadelphia, 6th edition, 1918, 517.

"Mercuric iodide	10 gm.
Potassium "	5 "
Sodium hydroxide	20 "
Water	100 cc.

The mercuric iodide is rubbed up in a small mortar with water, worked into a flask, and the potassium iodide added. The sodium hydroxide is dissolved in the remaining water, and the cooled solution is added to the above solution. After settling and decanting, the solution is kept in a dark bottle."

[9] Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 496.

The former error can be reduced to a minimum by observing all possible precautions to keep conditions uniform, such as the amount of reagent, temperature, time of standing before comparison, etc.; while the latter error may be decreased by increasing the number of readings. In all colorimetric work reported in this paper, six readings were made on each unknown and its standards. The error arising from matching of unequal column heights in the colorimeter was eliminated by preparing the "standard" and "unknown" of practically equal concentrations.

The standard Nessler reagent was retained for use with amounts of nitrogen of 0.5 mg. per 100 cc.[10] or less, since it is more sensitive, and in the presence of small amounts of nitrogen more stable than is the modification of Winkler.[11]

The Nessler-Winkler reagent was used according to the directions of Folin and Farmer.

By using these reagents with the above amounts of nitrogen and employing only redistilled water, there was not the slightest tendency towards the formation of cloudiness. The colors remained perfectly clear and manifested no tendency to precipitate even after several hours. The use of ordinary distilled water, freed from ammonia by bromine and caustic soda, for diluting the reagent and making the Nesslerized solution to the mark, as has been recommended by Folin and Denis,[12] did not prove satisfactory, as there was some tendency for cloudiness to form on the addition of the reagent.

For amounts of ammonia nitrogen of 0.5 mg. and less, using the standard Nessler reagent, the procedure was as follows: The

[10] The expressions "mg. N" and "mg. N per 100 cc." used in this paper in connection with Nesslerization refer to mg. of nitrogen contained in the colorimeter solution; *i.e.*, the solution after the addition of the Nessler reagent and dilution to the mark.

[11] This is probably due to the difference in the proportions of the mercuric salts and the potassium iodide in the two reagents. The sensitiveness increases with addition of mercuric salts and decreases by the addition of potassium iodide (see Olsen, J. C., Quantitative chemical analysis, New York, 5th edition, 1916, 412). Folin and Denis (Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 479) point out, however, that for satisfactory results with their modified reagent, it should contain more potassium iodide than does the standard Nessler's reagent.

[12] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 534.

desired amounts of ammonia were measured into 100 cc. graduated flasks from the ammonium hydroxide or ammonium sulfate standards by means of burettes. These solutions were then diluted to approximately 90 cc. with ammonia-free water, 2 cc. of Nessler's reagent were added, and the solutions were made to the mark. After 15 minutes, portions of the solutions were transferred to the colorimeter cylinders, and the colors compared, as mentioned above. The standard was set at 40 mm. and three of the six readings were made after adjusting the unknown from above, and three after adjusting from below.

For amounts of nitrogen equal to 0.5 mg. or more per 100 cc. requiring the Nessler-Winkler reagent, the procedure is the same except that the solutions are made to 70 cc. in the flasks, 25 cc. of the diluted reagent added; they are then made to the mark, and the colorimetric comparisons made against a 20 mm. standard 30 minutes after adding the reagent. Six readings were made as described above.

The desired amounts of ammonia nitrogen were obtained by using the following amounts of dilute solutions:

N_2	Solution.	Normality.
mg.	*cc.*	
0.10	10.0	N/1,400
0.50	50.0	N/1,400
1.00	10.0	N/140

For each amount of nitrogen, ten solutions (also ten standards[13]) were Nesslerized. Although the probable error of a method should be obtained by employing the results from a great many determinations using the procedure involved, the *order of magnitude* of the error of the procedure can be shown by using ten typical determinations of the procedure studied.

The probable error, r, of each individual observation is calculated with the aid of the formula

$$r = \pm 0.6745 \sqrt{\frac{\Sigma \Delta^2}{n-1}}$$

in which Δ indicates the deviation of each value from the mean, and n the number of observations. The results are shown in Table I.

[13] The ammonium sulfate solutions were taken as "standards," the hydroxide solutions as "unknowns."

The results show that the probable error increases with increasing amounts of nitrogen. This must unavoidably be the case since with the larger amounts of nitrogen the colors produced are intense, a low column height (± 20 mm.) must be used in the colorimeter, and very slight differences in readings result in marked differences in the value found for mg. of nitrogen.

Computations were next made to ascertain to what extent the errors in the colorimetric procedure as used lay in the readings themselves and to what extent in the color produced by the reagent.

TABLE I.

Probable Error of Nesslerization.

Determination No.	Nitrogen taken.		
	mg. 0.10	mg. 0.50	mg. 1.00
	Found.		
1	0.106	0.468	0.972
2	0.098	0.516	0.996
3	0.102	0.511	1.014
4	0.102	0.497	1.013
5	0.103	0.508	1.004
6	0.102	0.489	0.995
7	0.104	0.507	1.008
8	0.105	0.476	0.962
9	0.108	0.504	0.995
10	0.106	0.487	1.002
Average	0.103	0.496	0.996
r	±0.0019	±0.0107	±0.0114
r expressed as per cent	1.8	2.1	1.1

As stated above, each colorimetric determination is the average of six readings, and of course possesses an error, that of reading the colorimeter. That is, if we designate the value obtained by the colorimetric determination by a and its error by e the correct value is equal to $a \pm e$. In a series of determinations, we designate these values by $a_1 \pm e_1, a_2 \pm e_2, a_3 \pm e_3 \ldots\ldots a_n \pm e_n$. Where the values of a_1, a_2, a_3 are obtained by averaging a number of readings as was done in this case, the values of e_1, e_2, e_3, *i.e.* the probable error R_1, R_2, R_3 *of the average*, may be computed with the aid of the formula

$$R = \pm 0.6745 \sqrt{\frac{\Sigma \Delta^2}{n(n-1)}}$$

The values of R_1 to R_5 (e_1 to e_5) were computed for a_1 to a_5 of the determinations of 1 mg. portions, reported in Table I. The results appear in Table II.

Since the values obtained for R_1 to R_5 are in every case less than the value ± 0.0114 obtained for the probable error of a set of determinations, it follows that the color produced by the same amount of nitrogen is not perfectly concordant in a series of determinations.

The error in the colorimetric method for nitrogen then increases with increasing amounts of nitrogen, and is due in part to error in

TABLE II.

Probable Error of Arithmetical Mean of Colorimeter Readings on the Same Solution.

Reading No.	Determination No.				
	1	2	3	4	5
1	0.982	1.006	1.006	1.001	1.011
2	0.965	0.996	1.015	1.021	1.011
3	0.961	0.988	1.011	1.015	1.006
4	0.982	1.001	1.025	1.025	0.996
5	0.961	0.992	1.011	1.001	1.006
6	0.978	0.992	1.015	1.015	0.996

a_1 0.972, a_2 0.996, a_3 1.014, a_4 1.013, a_5 1.004.
R_1 ± 0.0028, R_2 ± 0.0018, R_3 ± 0.0016, R_4 ± 0.0028, R_5 ± 0.0019.

measurement, and in part to differences in the amounts of color produced by the reagent under the conditions of the determinations.

The Probable Error of Titration.

In order to avoid all error due to the solubility of the glassware commonly employed as titrimetric flasks, well seasoned Pyrex glass Erlenmeyer (300 cc.) flasks were used in determining the probable error of titration.

Standard ammonium hydroxide was run into the titration flasks from a burette, 25 cc. of 0.02 N acid were added, then sufficient neutral water was added to bring the volume to 100 cc., the con-

tents were boiled to expel carbon dioxide, and after cooling to 10–15° the titration was completed, using 0.02 N alkali. The error reported here includes the error of the burettes, but since this is less than that tolerated by the Bureau of Standards, the errors reported in Table III are essentially attributable to the titration alone.

TABLE III.

Probable Error of Ammonia Determination by Titration.

	Nitrogen taken.		
	mg. 0.100	*mg.* 0.500	*mg.* 1.00
	Found.		
	0.101	0.502	1.001
	0.098	0.505	1.002
	0.093	0.505	0.995
	0.098	0.505	1.001
	0.093	0.510	0.992
	0.099	0.508	1.001
	0.093	0.499	0.992
	0.101	0.508	1.005
	0.098	0.502	1.005
	0.093	0.502	1.006
Average	0.097	0.505	1.000
r	±0.0022	±0.0022	±0.0035
r expressed as per cent	2.06	0.38	0.35

The results of the two methods of ammonia determinations as summarized in Table IV show some interesting differences.

TABLE IV.

	Nesslerization.		Titration.	
N_2 taken.	Probable error.	Error.	Probable error.	Error.
mg.	*mg.*	*per cent*	*mg.*	*per cent*
0.10	±0.0019	1.80	±0.0022	2.06
0.50	±0.0107	2.10	±0.0022	0.38
1.00	±0.0114	1.10	±0.0035	0.35

The probable error of Nesslerization is variable and tends to increase with increasing amounts of ammonia while that of

titration remains practically constant. For quantities of nitrogen above 0.50 mg. portions, titration must be considered more accurate than Nesslerization. With considerably less than 0.50 mg. portions of ammonia nitrogen, Nesslerization is slightly more accurate than titration.

The Probable Error of Distillation and Titration.

It sometimes happens that the ammonia in an unknown may be determined directly by Nesslerization, whereas in the case of the titration procedure, the ammonia must always be transferred by distillation (or aeration) to a measured amount of standard acid. Indeed Folin and Denis,[14] and Folin and Bell[15] have recently proposed a series of very convenient methods in which the nitrogen is determined directly by Nesslerization. Thus the question arises as to the error of titration plus the error of distillation. Accordingly the following series of determinations was carried out.

The distilling apparatus used in estimating the probable error of distillation and titration has been previously described.[16] This apparatus is of Pyrex glass with the exception of one close rubber connection which was found not to vitiate the results when working with very small amounts of nitrogen. While this type of apparatus is not recommended for general analytical work, it has been found excellent for the most careful work where small differences must be determined. However, by using the devices described elsewhere,[17] together with good block tin condensers, and the modified Benedict procedure of distillation[18] very accurate results can be obtained with small amounts of nitrogen. The results obtained on 1 mg. of nitrogen are reported in Table V.

The probable error of distillation and titration is of the same order of magnitude as the probable error of titration alone. In other words the error of titration and distillation on a 1 mg. portion

[14] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473, 491, 497, 501, 505.

[15] Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

[16] Allen, E. R., and Davisson, B. S., *Ann. Missouri Bot. Gardens*, 1919, vi, 45.

[17] Davisson, B. S., *J. Ind. and Eng. Chem.*, 1919, xi, 465.

[18] Allen, E. R., *Ann. Missouri Bot. Gardens*, 1919, vi, 23.

is less than that accompanying direct Nesslerization of an equal amount.

Although no data are included here for larger amounts of nitrogen, the probable error remains almost constant in absolute value and decreases in percentage with larger amounts of nitrogen. Considerable data on more complicated apparatus and with larger amounts of ammonia nitrogen showed but little greater error.[19]

TABLE V.

Probable Error of Distillation and Titration.

Nitrogen taken.
mg 1.00
Found.
1.005
0.996
1.010
0.998
1.013
1.002
0.996
1.007
1.005
1.005
Average 1.0037
r ..... ±0.0038

Preparation, Preservation, and Use of 0.02 N Solutions.

Since more precaution must be taken in the use of 0.02 N solutions than is usually observed in volumetric analysis, it seems worth while to record at this point the procedures which, after extended trial, have proved satisfactory.

As an acidimetric standard we have found sodium carbonate satisfactory from the standpoint of convenience and accuracy. It has the advantage that the standardization is carried out under conditions practically the same as are encountered in the regular work.

[19] Allen, E. R., *J. Ind. and Eng. Chem.*, 1915, vii, 521. Davisson, B. S., Allen, E. R., and Stubblefield, B. M., *ibid.*, 1916, viii, 896. Davisson, B. S., *ibid.*, 1918, x, 600; 1919, xi, 465.

The sodium carbonate is prepared from pure sodium bicarbonate by heating to 270–300° in platinum until constant weight is obtained. The bicarbonate is easily prepared in a high degree of purity, either by recrystallizing of a high grade product,[20] or by carbonating a filtered concentrated solution of normal carbonate.

It has been our practice never to prepare solutions *exactly* 0.02 N. The volumetric adjustment of large volumes of solutions is not entirely trustworthy unless the final solution is restandardized. Where logarithmic computations are used nothing is to be gained by having the solutions of an exact fraction of normality unless perhaps when they are used for a variety of purposes. Moreover, in the process of standardization no attempt is made to weigh out an exact amount of a molar weight of sodium carbonate, since such a procedure is inconvenient and, because of the prolonged exposure of the powdered material to the air, is likely to be inaccurate.

20 or 25 cc. portions of approximately 0.02 N acid are titrated each time. The sodium carbonate equivalent to such quantities of solutions is a rather small amount to weigh with extreme accuracy. This difficulty is, however, readily avoided by the method of aliquoting which has been recommended by Eastlack.[21] The strength of the acid is expressed as its *nitrogen titer; i.e.*, its *nitrogen equivalent in gm. (or mg.) per cc.* The computation is easily made with the aid of logarithms.

It has been our practice to prepare 8 to 10 liter portions of solutions at one time. The reservoir bottles are provided with soda-lime guard tubes and the solutions are supplied to the burettes preferably through siphon tubes of Pyrex glass. The acid solution does not change strength appreciably on standing. In Table VI are given the results of change in concentration of a solution of approximately 0.02 N H_2SO_4. The original volume was 8 liters.

The sodium carbonate was in all cases prepared from Kahlbaum's bicarbonate "Zur Analyse." In Standardization I the bicarbonate was recrystallized, while in the subsequent standardizations this step was omitted. The variations in the first four determinations are insignificant. The higher value reported in

[20] Olsen, J. C., Text-book of quantitative chemical analysis, New York, 5th edition, 1916, 259.

[21] Eastlack, H. E., *J. Am. Chem. Soc.*, 1918, xl, 620.

the last standardization may have been due to the fact that when the standardization was made, less than 1 liter remained in the bottle, and the walls of the bottle were covered with water which had vaporized and condensed.[22] The maximum variation, however, between the lowest and highest value in the above standardizations amounts to only 0.0018 mg. of nitrogen per 1 cc. or 0.045 mg. per 25 cc. of acid, an error therefore of less than 1 per cent, and which may be disregarded except in the most exact work. Another large bottle of acid, which had been in use for a little over 11 months, showed a nitrogen titer of 0.2835 mg. at the beginning and 0.2850 at the end of this period.

The question of the deterioration of $\pm$ 0.02 N alkali solution was also studied. In Table VII are given the results of the determinations at different intervals.

TABLE VI.

Standardizations of Approximately 0.02 N H_2SO_4.

Standardization No.	Date.	Nitrogen titer.
I	Nov. 19, 1917	0.2825
II	" 13, "	0.2827
III	Dec. 17, "	0.2833
IV	Jan. 23, 1918	0.2833
V	Mar. 26, "	0.2843

From this it is seen that the change in strength of the alkali solution in 1 month is quite appreciable. It has become our practice to check the alkali solution every week. No attempt has been made to prepare or keep the alkali exactly equivalent to the acid since the cc. of alkali obtained in any titration is readily converted into cc. of alkali exactly equivalent to the acid with the aid of the equation.

$$\text{Log } c = \log A - \log \frac{\text{cc. of alkali}}{\text{cc. of acid}} \left(\text{or} + \log \frac{\text{cc. of acid}}{\text{cc. of alkali}} \right)$$

where c = cc. of alkali of exactly the same strength as the acid and A = the observed cc. of alkali. The ratio $\left(\frac{\text{cc. of alkali}}{\text{cc. of acid}}\right)$ is, of course, determined in previous titrations.

[22] It has subsequently been found that a layer of white paraffin oil on the surfaces of the solutions prevents loss from evaporation.

In the use of 0.02 N solutions certain precautions are naturally necessary which may be disregarded in the more concentrated solutions. We have found that neglecting any of the following measures introduces an appreciable error.

1. The volume of solution in the titrating flasks should be kept small and constant (*i.e.* within 100 to 125 cc.), and should be well cooled and free from carbon dioxide. This procedure reduces the error from the hydrolysis of the indicator salt to a small and constant magnitude, and insures a sharp and certain end-point.

2. An approximately constant amount of standard acid should be used in each titration in order to avoid any "salt effect" on the indicator. Thus, if the ratio between the acid and the alkali is established on 25 cc. portions and 50 cc. of acid are used in a determination, a slight error is introduced.

TABLE VII.

Change in Strength of ± 0.02 N Alkali.

Determination No.	Date.	Alkali equivalent to 25 cc. of acid.
I	Nov. 9, 1917	25.72
II	Dec. 14, "	25.55
III	Jan. 16, 1918	25.47
IV	Feb. 14, "	25.34
V	Mar. 20, "	25.16

3. Standard solutions must be properly prepared and their strength determined in the dilution in which they are finally used.

4. Solutions which have stood 12 hours or more in the burettes must be discarded.

5. The titration flasks must be of practically insoluble glass. Pyrex glass is the only material (except transparent quartz) which we have found to be entirely satisfactory.

DISCUSSION.

From the results reported above, which represent it is believed a fair measure of the relative accuracies of the titrimetric and colorimetric methods for determining ammonia, it seems that the latter are slightly more accurate. Both types of determinations have been made, it is true, under conditions more nearly ideal than

one can attain in general practice, yet the conditions described for accurate titrations may be more nearly attained than can those for satisfactory Nesslerization. Titrimetric procedures have the added advantage over the colorimetric in that they are applicable to a wide range of nitrogen values, and hence by a regulation of the size of the sample the error due to aliquoting may be kept smaller. For example, if we have a quantity of material for analysis containing 10 gm. of nitrogen, $\frac{1}{10,000}$ of the material would be taken for the determination by the colorimetric procedure. If our readings show 1.00 mg., the value is obviously 1.00 ± 0.0114 mg., and the amount of nitrogen in the material sampled would be between the limits of 10.114 and 9.886 gm. Only the first digit to the right of the decimal would be of any significance. On the other hand, if the titrimetric method is used, $\frac{1}{2,000}$ of the material may be taken for analysis. If our determination shows a value of 5.000 mg., the true value is 5.000 ± 0.0038 mg., and our final value lies between the limits 10.008 and 9.992, in which case the first digit to the right of the decimal is significant, the second doubtful, and the third of no significance. Expressed in another way the error in the colorimetric and titrimetric procedures would be under the best conditions 0.228 and 0.015 gm. respectively.

The disadvantage of the colorimetric determination of ammonium nitrogen is that the reaction involved is imperfectly understood, and it is not surprising, therefore, that in spite of the different modifications of the Nessler reagent which have appeared, the appearance of clouds and precipitates cannot be avoided with certainty by different workers.

Aside from this point, it is our opinion that the colorimetric methods are, even under the best conditions, more subject to personal error than are volumetric or titrimetric procedures. The accurate measurement of color is a more elusive and difficult operation than is generally believed and many colorimetric procedures are quite faulty in theory.

Some of the more common sources of error will be briefly considered. One which may become quite appreciable in routine colorimetric work (but which was avoided in the determinations reported above) is that due to unequal column heights, especially if the procedure of setting the standard at a definite height and

adjusting the unknown is too rigidly followed. The fact must not be lost sight of that the ordinary equation of colorimetry

$$c_1 = c_2 \frac{h_2}{h_1}$$

is based on the incorrect assumption that the solvent has no effect on the passage of light. If this error is reduced to a negligible magnitude either by the preparation of a large number of standards or by the scrupulous filtering of all solutions, as has been recommended by Krüss and Krüss[23] for colorimetric procedures in general, then the procedure becomes distinctly time-consuming.

It is of interest to note in this connection that Dehn[24] in a recent paper on "Fallacies in colorimetry," after pointing out the unsoundness of many existing procedures, concludes that in Folin's method of creatinine estimation, by way of illustration, there are twelve probable sources of error. A similar array also exists in the colorimetric determination of ammonium nitrogen. These fundamental errors of colorimetry probably account for the skepticism with which many chemists are inclined to view colorimetric procedures.

In view of these points we have therefore adhered to the titrimetric procedures in our work on nitrogen methods.

While the colorimetric procedures for the determination of nitrogen have been of great service because of their brevity, it should be recognized that except when dealing with amounts of nitrogen less than a few tenths mg., titrimetric methods are capable of greater accuracy and should be chosen where exact results are required.

[23] Krüss, G., and Krüss, H., Kolorimetrie und quantitative Spektralanalyse in ihrer Anwendung in der Chemie, Hamburg, 1909, 31.

[24] Dehn, W. H., *J. Am. Chem. Soc.*, 1917, xxxix, 1392, 1399.

A STUDY OF CREATINURIA IN INFANTS.

I. RELATION OF CREATINURIA TO ACIDOSIS. THE ELIMINATION OF INGESTED CREATINE AND CREATININE.

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Creatine apparently never appears in the urine of a normal adult male in appreciable amounts. Normal women[1] excrete creatine periodically, while infants and children[2] exhibit a creatinuria regularly on normal diets. Creatine metabolism must, therefore, be studied in both sexes, and at different stages of growth, if a complete set of facts regarding the processes concerned is to be obtained. We have undertaken to put to experimental test in the male infant certain factors supposed to bear relation to creatinuria. We have also made measurements of the elimination of ingested creatine and creatinine by infants, there being no quantitative data at hand on this point at this age.

Methods.

The subjects used for the experiments described in this and the succeeding paper were infants obtained from a well conducted home for foundlings. One of them, Subject G, was a normal infant. The others failed to come up to the specifications for normal infants only in the respect that they were more or less underweight. There was in no instance a history of recent nutritional disturbance, or, during the experimental periods, symptoms

[1] Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293. Rose, M. S., *J. Biol. Chem.*, 1917, xxxii, 1.

[2] Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 265. Folin, O., and Denis, W., *ibid.*, 1912, xi, 253.

of malnutrition. They all made moderate or rapid gains in weight on the experimental diets. The diet was in all cases cow's milk, the various modifications of which, as regards the protein, butter fat, and lactose content, are indicated in Tables I and II. The quantity of food given was carefully measured and all of it was taken. There was always a foreperiod of several days on the experimental diet before the collection of urine specimens was begun.

The urine collection periods were of 2 or more days duration. The infant during this time was immobilized by means of a comfortably arranged metabolism frame. The urine was preserved with 5 per cent thymol in chloroform. The bottle receiving the urine was stoppered and shaken every 4 hours to insure thorough disinfection, and at the end of 12 hours was replaced by a second bottle and put in an ice box. The regularly close agreement of the day to day figures indicates the quantitatve collections obtained.

Creatinine and creatine were determined by the methods of Folin.[3] Conversion of creatine was obtained by autoclaving the urine samples, 5 to 10 cc., at 130° for 18 to 20 minutes with 1 cc. of N hydrochloric acid. The acid was neutralized by adding an equivalent of 10 per cent sodium hydroxide before developing the color. Total nitrogen in milk and urine was determined by the Kjeldahl method. The factors of acid excretion in the urine, hydrogen ion concentration, titratable acidity, and ammonia, were measured as directed by Palmer and Henderson.[4]

Relation of Acidosis to Creatinuria.

One gathers from the literature the impression that acidosis stands accepted as a cause of creatinuria, or, at least, as a factor in its production. It is our belief that the presence of an actual acidosis has been in most instances only a matter of inference, and, furthermore, that in conditions where acidosis and creatinuria undoubtedly do occur together evidence of a causal relationship is lacking.

[3] Folin, O., *J. Biol. Chem.*, 1914, xvii, 469.

[4] Palmer, W. W., and Henderson, L. J., *Arch. Int. Med.*, 1913, xii, 153.

Krause and Cramer,[5] in 1910, pointed out that creatinuria occurs in all conditions which lead to an acidosis. They cited chiefly the creatinuria of starvation and of diabetes. At that time, however, the fallacy of regularly inferring an acidosis from the presence in the urine of ketone acids was prevalent. Since we know now that production of ketone acids is only occasionally of such degree as to cause an acidosis it is obviously incorrect to regard the occurrence together of ketonuria and creatinuria as proof of the relation of the latter to acidosis.

Underhill[6] has published results of experiments with rabbits, designed to show a relation between creatinuria and diets which produce an acid urine. An acid urine was produced by giving the rabbits a grain diet composed of corn and oats. On this diet, creatine was regularly found in the urine. When carrots were given, instead of the grain diet, creatine was found in the urine in greatly diminished quantity, or was absent. Underhill considered these results as proof of a relationship between creatinuria and acidosis. The presence of acidosis "was considered indicated when the urine of the rabbit became strongly acid, as shown by determination of the hydrogen ion concentration. . . . . In the sense of the term acidosis as used here, that is, a condition of alkali depletion, the assumption given above is undoubtedly correct since ordinarily the rabbit secretes urine which is strongly alkaline." Since rabbits cannot provide the base ammonia in compensation for an unusual acid production, it is probable, though not definitely proven by the acid urine, that the rabbits Underhill experimented with did develop an acidosis.

From the well established facts in regard to the process of acid excretion in man, it is absolutely incorrect to assume a depletion of fixed alkali from an unusually acid urine. The neutrality mechanism in man is remarkably extensible and is capable of neutralizing and conveying into the urine unusually large amounts of acid without disturbance of the acid-base equilibrium within the body. The gross adjustment for an unusual acid production is an increase in production of ammonia. The fine adjustment, by means of which the reaction of the blood is maintained at the

[5] Krause, R. A., and Cramer, W., *J. Physiol.*, 1910, xl, p. lxi.
[6] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127, 141.

TABLE I.

Effect of Ingested Alkali and Acid upon Output of Urinary Creatine.

Experiment No.	Date.	Protein per day.	Urine. Volume.	Total N.	pH	Titratable acid 0.1N.	NH_3 0.1N.	Total acid 0.1N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Remarks. Diet.
Subject G, age 10 months; weight 6.5 to 7.1 kg.												
	1917	*gm.*	*cc.*	*gm.*		*cc.*	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*	
1 Control period.	Mar. 17	20	500		6.4	44	125	169		78		Milk, 600 cc.
	" 18	20	550		6.4	41	130	171		76		Barley water, 300 cc.
	" 19	20	500		6.4	36	121	157		77		Lactose, 40 gm.
	" 20	20	490		6.5	32	118	150		79		
	Averages..	20	510	1.89	6.4	38	124	162	137	78	59	
Experimental period.	Mar. 21	20	500		8.0	−43	32		137	82	55	Same food + 4 gm. of $NaHCO_3$.
	" 22	20	490		8.2	−39	40		133	82	51	Same food + 4 gm. of $NaHCO_3$.
	" 23	20	540		8.1	−40	40		137	81	57	Same food + 4 gm. of $NaHCO_3$.
	" 24	20	470		8.3	−40	35		135	84	51	Same food + 4 gm. of $NaHCO_3$.
	Averages..	20	500	1.97	8.2	−41	37	−4	136	82	54	
2 Control period.	May 7	30	490		6.2	100	240	340	187	82	105	Whole milk, 900 cc.
	" 8	30	490		6.4	90	204	294	175	81	94	Lactose, 20 gm.
	Averages..	30	490	4.37	6.3	95	222	317	182	82	100	

Experimental period.	May 10	30	570		7.6	−100	92		191	83	109	Began $NaHCO_3$ 36 hrs. before and continued through May 10. 4 gm. per day.
	" 11	30	520		7.6	−25	92		190	82	108	No $NaHCO_3$ given.
	Averages. .	30	545		7.6	−65	92	27	191	83	109	
3 Control period.	May 21	12	590		6.4	36	95	131	116	65	51	16 per cent cream, 390 cc.
	" 22	12	500		6.4	30	91	121	100	60	40	Water, 730 cc.
	" 23	12	540		6.4	37	76	113	112	63	49	Lactose, 50 gm.
	Averages. .	12	543	1.20	6.4	34	86	120	110	63	47	
Experimental period.	May 25	12	590		5.9	52	100	152	116	61	55	Administered 100 cc. of 0.1N HCl.
	" 26	12	600		5.9	60	119	179	116	63	53	No acid given.
	Averages...	12	595	—	5.9	56	109	165	116	62	54	
Subject M, age 14 months; weight 6.5 kg.												
4 Control period.	May 21	7	690		6.2	30	90	120	130	70	60	16 per cent cream, 230 cc.
	" 22	7	640		6.2	32	94	126	136	72	64	Water, 900 cc.
	" 23	7	650		6.2	33	84	117	133	70	63	Lactose, 30 gm.
	" 24	7	620		6.2	31	92	123	130	68	62	
	Averages...	7	650	0.84	6.2	32	90	122	132	70	62	
Experimental period.	May 27	7	680	—	8.2	−32	31	−1	131	71	60	4 gm. of $NaHCO_3$ daily; begun May 26.

normal pH is managed by excretion of the phosphates in correct relative amounts. Slight variations in acid production may be entirely compensated by variation in the relative amounts of the phosphates excreted, the ammonia factor remaining stationary. The acidity of the urine on normal diets may, for this reason, vary widely, the pH being frequently as great as when acidosis is present. It is therefore impossible to obtain from the pH of the urine a dependable indication of the presence of an acidosis.

It is equally incorrect to assume that diets containing an unusually large amount of acid-producing elements, or which lead to the production of abnormal acids, will necessarily cause an acidosis. Acidosis cannot safely be considered indicated unless a depletion of the fixed alkali of the blood has been demonstrated. Denis and Minot[7] have been able to obtain with women subjects an excretion of creatine by giving diets unusually high in protein; which necessarily contain a large quantity of acid-forming elements. They have found that the addition of sodium bicarbonate to this diet produces no appreciable effect on the creatinuria. This result shows very clearly that a wide variation in the acid-base value of a diet may bear no relation to creatine excretion.

We have thought it worth while to illustrate this point further by adding acid or base to the food of infants. The results of these experiments are tabulated in Table I. We present the results of a single experiment in which 100 cc. of 0.1 N hydrochloric acid were given to an infant in its food, in divided amounts, during the course of 24 hours. There is a well marked effect on the factors concerned in acid excretion during the day the acid was given, and also on the day following. The value for creatine excretion remains the same as during the control period. We gave sodium bicarbonate freely in several instances, but did not find a change in the quantities of creatine eliminated.

We have been able to find a record of but one set of experiments in which creatine was measured in the presence of a demonstrated acidosis. Sawyer, Stevens, and Baumann,[8] using as subjects male children, were able to produce quite regularly a depletion of the fixed alkali of the blood by giving a high fat and low carbo-

[7] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxvii, 245.

[8] Sawyer, M., Stevens, F. A., and Baumann, L., *Am. J. Dis. Child.*, 1918, xv, 1.

hydrate diet. They found in all their experiments that during the period of acidosis there was a well marked increase in the excretion of creatine. They were also usually able to obtain a lowering of the bicarbonate of the blood by giving a low calorie diet, with nearly always an increase in creatine excretion. They found, however, in three instances an increase in creatine excretion on the low calorie diet without a reduction of blood bicarbonate. Their results suggest a relationship of acidosis to creatinuria. Such an inference cannot, however, be drawn with certainty because of the wide variation in the diet of factors other than those related to acid production. The authors themselves express their belief that the creatinuria was not due to the acidosis *per se*. We would suggest that the increase in creatine excretion may have been due to an abnormal protein metabolism, caused, in one case, by an inadequate intake of protein and, in the other, by the absence of the protein-sparing effect of carbohydrate. As compared with its value on the normal control diet, the nitrogen in the urine was relatively high during the low calorie periods and absolutely high during the high fat intake periods.

We would say in conclusion that variations in the acid-base value of normal diets cannot possibly bear relation to creatinuria in man for the reason that they are never of sufficient magnitude to disturb the normal acid-base equilibrium within the body. As regards the relation to creatine excretion of acidosis produced by abnormal diets, we do not find in the literature satisfactory evidence indicating acidosis *per se* as a factor concerned in causing or increasing creatinuria.

The Elimination of Ingested Creatine and Creatinine in Infants.

It is well established by the original investigations of Folin[9] and of Klercker[10] that large amounts of creatine may be ingested by adult men with a failure of creatinuria. Moreover, doses of creatine, which fail to produce creatinuria on a low protein diet, cause a creatine output on a high protein intake. Only a small percentage of the ingested creatine appears in the urine

[9] Folin, O., Festschrift für Olof Hammersten, Upsala, 1906.

[10] af Klercker, K. O., *Beitr. Chem. Physiol. u. Path.*, 1906, viii, 59; *Biochem. Z.*, 1907, iii, 45.

in any case, and for the most part, the ingested nitrogen remains unaccounted for in the urine.

Mellanby[11] suggested that the results given above may be due to the action of intestinal bacteria in destroying the ingested creatine. In fact, Twort and Mellanby[12] have isolated a bacillus from the human feces which destroys creatine. Hence, according to these authors, in creatine-feeding experiments this factor must be taken into account. While the validity of this observation cannot be denied, it must be pointed out that according to more recent investigations, in which the creatine is introduced parenterally, the original contention of Folin and of Klercker is confirmed. Lyman and Trimby[13] find that 76 per cent of the creatine nitrogen, injected subcutaneously into man, remains unaccounted for in the urine. Meyers and Fine,[14] and Rose and Dimmitt,[15] after ingestion, as well as Lyman and Trimby[13] after injection of creatine, believe that they found an increased output of urinary creatinine which they attribute to converted creatine. In this finding they stand in opposition to the results of Folin[9] and of Klercker.[10]

When creatinine is fed to adult men, observers are agreed that greater amounts are eliminated than is the case with creatine. Folin[9] and Rose and Dimmitt[15] report recovery of 80 per cent of the creatinine ingested.

Krause[16] investigated the fate of exogenous creatine in two girls (aged 6 and 11 years), and two boys (aged 5 and 8 years); the older of each sex was not excreting creatine. He finds that, in children, relatively small amounts of creatine (0.3 to 0.35 gm.) lead to an increased creatinuria if one already existed, or to a creatinuria where there was none previously. The author concludes that in children the power of "assimilating" creatine, if measured in absolute amounts, is less developed than in adults. Further, this is true even when the power of "assimilating" creatine is calculated per kilo of body weight; and the younger the child the less ingested creatine can it retain.

[11] Mellanby, E., *J. Physiol.*, 1907–08, xxxvi, 447.
[12] Twort, F. W., and Mellanby, E., *J. Physiol.*, 1912, xliv, 43.
[13] Lyman, J. F., and Trimby, J. C., *J. Biol. Chem.*, 1917, xxix, 1.
[14] Meyers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 377.
[15] Rose, W. C., and Dimmitt, F. W., *J. Biol. Chem.*, 1916, xxvi, 345.
[16] Krause, R. A., *Quart. J. Exp. Physiol.*, 1914, vii, 87.

The investigation to be presented herewith was instituted in order to test the effect of ingested creatine upon the creatinuria of infants, not only because of the interest attached to the data thus obtained, but also since they are necessary in connection with results presented in the paper which follows.

Both subjects used were of the male sex and creatine was a constant urinary constituent. The creatine and creatinine administered were dissolved in the total amount of milk to be fed during the day. Hence, the subject received but a small amount of creatine or creatinine at each feeding.

The creatine was a pure preparation free from creatinine and contained 1 mol of water of crystallization. The amounts noted in the protocols, however, are calculated as anhydrous. The creatinine was likewise a pure preparation isolated from urine.

EXPERIMENTAL.

In the experiments detailed in Table II, it is apparent that even on a low protein diet (Experiments 1 and 5), from 12 to 16 mg. per kilo, or an absolute amount of 88 mg., suffice to cause an increased urinary creatine. No attempt is made to determine the least amount of ingested creatine which would lead to such an increase, but indications are that it would be very small.

As regards the completeness of excretion, it will be noted that in Experiments 1, 2, and 5, on a low protein diet, 24 to 34 per cent of the ingested creatine is recovered in the urine during the first 24 hours. It must be recalled that, in these experiments, the creatine administered was mixed with the food and given throughout the day with each feeding. As a result it is found that the urine of the 2nd day still contains extra creatine. Hence, if the total extra creatine excreted in 2 days is calculated, we obtain figures of from 35 to 58 per cent.

In Experiment 3, the subject is on a high protein level. The afterperiod is considerably lengthened in order to study more completely the lag in creatine excretion. In this experiment, taking the 1 day foreperiod as the normal output, in the first 24 hours there is an extra excretion of 52 per cent of the creatine ingested. Moreover, it will be seen that the urinary creatine continues to be high for at least 3 days after the day of feeding.

TABLE II.

Effect of Ingested Creatine and Creatinine upon Urinary Creatine and Creatinine.

Experiment No.	Date.	Weight.	No. of days on diet.	Protein per day.	Urine.					Remarks.
					Total N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Creatine.	Diet.
Subject G, age 10 months.										
1 Control period.	*1917* May 31	*kg.* 7.2	18	*gm.* 12	*gm.* 1.57	*mg.* 136	*mg.* 71	*mg.* 65	*mg.* 75	Low protein diet { 16 per cent cream. water. Urine contained acetone during 3 days of experiment.
Experimental period.	June 1			12	—	156	71	85	99	Administered in food 0.088 gm. of creatine. Extra creatine excreted on 1st day = 24 mg. = 27 per cent of ingested creatine.
	" 2			12	—	143	72	71	82	Total extra creatine excreted in 2 days = 31 mg. = 35 per cent of ingested creatine.
2 Control period.	June 9	7.7	26	12	0.84	137	75	62	72	Low protein diet { 16 per cent cream. lactose. water.
Experimental period.	June 10			12	—	215	75	140	162	Administered in food 0.264 gm. of creatine. Extra creatine excreted on 1st day = 90 mg. = 34 per cent of ingested creatine.
	" 11			12	—	176	76	100	116	Total extra creatine excreted in 2 days = 134 mg. = 51 per cent of ingested creatine.

3 Control period.	June 25	8.2	9	40	5.16	194	87	107	124	High protein diet { whole milk.
Experimental period.	June 26			40	—	317	93	224	260	Administered in food 0.264 gm. of creatine. Extra creatine excreted on 1st day = 136 mg. = 52 per cent of ingested creatine.
	" 27			40	—	242	86	156	181	Total extra creatine excreted in 2 days = 193 mg. = 73 per cent of ingested creatine.
	" 28			40	—	233	95	138	160	Total extra creatine excreted in 3 days = 229 mg. = 87 per cent of ingested creatine.
	" 29			40	—	222	89	133	154	Total extra creatine excreted in 4 days = 259 mg. = 98 per cent of ingested creatine.
	" 30			40	—	204	88	116	135	Total extra creatine excreted in 5 days = 270 mg. = 102 per cent of ingested creatine.
4 Control period.	June 19	8.2	2	40	5.30	218	83	135	157	High protein diet { skim milk.
	" 20			40	—	216	84	132	153	
Experimental period.	June 21			40	—	314	185	129	150	Administered in food 0.2 gm. of creatinine. Extra creatinine excreted 1st day = 101 mg. = 50 per cent of ingested creatinine.
	" 22			40	—	234	107	127	147	Total extra creatinine excreted in 2 days = 124 mg. = 62 per cent of ingested creatinine.

TABLE II—*Concluded.*

Experiment No.	Date.	Weight.	No. of days on diet.	Protein per day.	Urine.					Remarks.
					Total N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Creatine.	Diet.
Subject S, age 8 months.										
5 Control period.	*1917* June 4	*kg.* 5.4	3	*gm.* 6	*gm.* 0.50	*mg.* 68	*mg.* 56	*mg.* 12	*mg.* 14	Low protein { 16 per cent cream. lactose. water.
	" 5			6	—	68	56	12	14	
Experimental period.	June 6			6	—	84	54	30	35	Administered in food 0.088 gm. of creatine. Extra creatine excreted on 1st day = 21 mg. = 24 per cent of ingested creatine.
	" 7			6	—	91	53	38	44	Extra creatine excreted in 2 days = 51 mg. = 58 per cent of ingested creatine.

These increases are greater than the experimental variations from day to day. In the first 2 days 73 per cent of the ingested creatine is excreted, in 3 days 87 per cent, and in 4 days 98 per cent. The figure on the 5th day is still slightly higher than the foreperiod, so that the total excretion is 102 per cent. Allowing 10 per cent, a very liberal allowance, for a possible variation in the normal excretion, the figure for creatine on the 5th day may be considered as a return to the normal. Therefore, the average normal excretion is between 124 and 135 mg. (130 mg.). On this basis we get a total extra excretion of creatine of 235 mg., or 89 per cent of the amount ingested.

Experiment 4 presents an isolated experiment on creatinine ingestion. In a period of 2 days, 62 per cent of the ingested creatinine appears in the urine. The daily output during this time is still not down to the normal figure of the foreperiod, hence it is possible that, had the period been lengthened, more creatinine might have been excreted.

These experiments show no evidence of the conversion of ingested creatine into creatinine or *vice versa*.

DISCUSSION.

The results given above show: first, that in the infant small amounts of ingested creatine lead to an increased urinary output. Second, there is evidence that in infants the ingested creatine is nearly or completely eliminated during a period of several days. Third, from a comparison with experiments in the literature of the behavior of creatine ingested by adult men with the behavior in the infants studied by us, the following points are suggested: (*a*) smaller absolute amounts of ingested creatine lead to urinary excretion of creatine in infants than is the case with adult males; (*b*) ingested creatine is more completely excreted by the infant than by the adult male; (*c*) although the comparison presents greater difficulties of demonstration, there is an indication that, per kilo of body weight, smaller quantities of ingested creatine lead to excretion of creatine in infants than in adult males.

In Experiments 1 and 5 (Table II), where the lowest protein level obtains, it is found that from 12 to 16 mg. of creatine per kilo of body weight or an absolute amount of 88 mg. is sufficient

to cause an increased urinary creatine of from 35 to 58 per cent in 2 days. Under similar conditions in the adult male, Folin[9] found it necessary to administer 6 gm. (in three portions) before excretion of creatine is obtained, and even then but 16 per cent of the amount ingested is recovered. Klercker[10] took 2.06 gm. (22 mg. per kilo) of creatine on a low protein diet with failure of excretion. Plimmer, Dick, and Lieb[17] are compelled to administer 2.5 gm. of creatine to an adult man, on an average protein intake, before creatinuria occurs. This amounts to 34.7 mg. per kilo of body weight.

On a high protein level (Experiment 3), the excretion in our subject is somewhat greater in an equal interval of time than on a low level of protein intake. Of the ingested creatine (264 mg. or 32 mg. per kilo of body weight), 73 per cent is eliminated in 2 days, and nearly, if not all, in an interval of 5 days. On a high protein level Folin[9] found that ingestion of 5 gm. of creatine (in three portions) leads to the excretion of 36 to 54 per cent of the amount ingested. Klercker finds that under like conditions when 2.59 gm. are taken (28 mg. per kilo) but 30 per cent of the ingested creatine is excreted.

The results obtained by us are in accord with those obtained by Krause[16] on older children, although this author did not observe the complete excretion of creatine which is indicated in our experiment.

In the normal adult (Folin,[9] Klercker,[10] and Meyers and Fine[14]), there is no such lag leading to a complete excretion of creatine as is observed in Experiment 3.

Powis and Raper [18] found that upon feeding 206 mg. of creatine to a girl 4 years old, with amyotonia congenita, there was an increased creatine excretion for 48 hours following its administration, leading to a nearly total elimination of the ingested creatine.

Determinations of creatine tolerance for children and adults of both sexes have not been made, and from scattered observations in the literature, accurate comparisons are impossible. However, on the basis of our experiments, it seems justifiable to say that

[17] Plimmer, R. H. A., Dick, M., and Lieb, C. C., *J. Physiol.*, 1909–10, xxxix, 98.

[18] Powis, F., and Raper, H. S., *Biochem. J.*, 1916, x, 363.

in the infant the tolerance is much less than in the adult, and, furthermore, excretion is more complete in the infant than in the adult.

The significance and bearing of these results on recent work will be brought out in the next paper.

CONCLUSIONS.

1. Variations in the acid-base intake have no effect on the creatinuria of infants.

2. There is no satisfactory evidence that acidosis *per se* is a factor in the production of creatinuria.

3. Small amounts of ingested creatine lead to an increase of the creatinuria in infants. In a single experiment, on a high protein diet, a practically complete elimination of ingested creatine was observed.

4. There is evidence that the infant differs radically from the adult in its behavior towards ingested creatine.

A STUDY OF CREATINURIA IN INFANTS.

II. RELATION OF PROTEIN INTAKE TO URINARY CREATINE.

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Effect on Creatine Excretion of Different Levels of Protein Intake Obtained by Giving Milk or Milk Dilutions.

The relation to creatinuria of various dietary factors has been suggested and investigated. Recent investigations seem to indicate quite definitely a relation of creatinuria to the size of the protein intake.[1] In as far as these observations apply to infants, investigators are confronted with the difficulty of administering a creatine-free diet. We shall present data in this paper which show that, in the infant on a milk diet, the protein intake *per se* is not entirely responsible for the increased output of creatine obtained by increasing the intake of milk.

One of us[2] several years ago, during a study of the nitrogenous end-products found in the urine of an infant at different levels of protein intake, noted that during the period when the least amount of protein was given there was an almost complete disappearance of creatine from the urine. As the quantity of protein in the food was increased, the amount of creatine in the urine rose steadily. The creatinine excretion preserved throughout the experiment a closely constant value. During all the experimental periods the infant received a normal caloric intake, and the nitrogen balance was positive. The size of the protein intake, as a factor concerned in determining the quantity of creatine in the urine,

[1] Denis, W., *J. Biol. Chem.*, 1917, xxx, 47. Denis, W., and Kramer, J. G., *ibid.*, 189. Denis, W., and Minot, A. S., *ibid.*, 1917, xxxi, 561.

[2] Talbot, F. B., and Gamble, J. L., *Am. J. Dis. Child.*, 1916, xii, 333.

seemed clearly indicated by this experiment. Folin and Denis[3] had previously suggested that this effect might be obtained in children by varying the quantity of protein given. They based their assumption on the theory that the normal creatinuria of children is due to a much higher level of protein consumption in proportion to body mass than is the case with adults.

We undertook some time ago to confirm these findings by repeating the experiment with several infants. Soon after we had begun our work, Denis and Kramer[1] published the results of similarly devised experiments, using as subjects several children and one infant. They found a wide difference in the amount of creatine in the urine when the excretion on high and low protein diets was compared. They conclude, "that the amount of creatine found in the urine of children is directly dependent on the intake of protein, being high when large quantities of protein (creatine-free) are ingested, decreasing and in some cases disappearing entirely when the child is fed a diet of an extremely low protein content." The protein constituents of the high protein diet given the children were eggs, cheese, gelatin, and milk. A mixture of whole and fat-free milk constituted the high protein food of the infant. During the low protein period, the infant received 40 per cent cream, oatmeal water, and lactose.

In the experiments on infants, to be reported in this paper, different levels of protein were obtained in quite the same way, except that for the low protein periods we used a dilution of 16 per cent cream. We therefore did not feed so small a quantity of protein as did Denis and Kramer,[1] which possibly accounts for the fact that we did not obtain in any instance a complete disappearance of creatine from the urine. A proper caloric value was obtained in the cream dilutions by the addition of lactose. The methods employed have been described in the preceding paper.

Results of Experiments.

The results of our experiments are given in Table I. They are substantially in agreement with those of Denis and Kramer.[1] A high excretion on whole milk and a low excretion on diluted cream is obtained in both instances. Dilutions of whole milk give an

[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 253.

TABLE I.

Effect on Creatine Excretion of Increasing the Quantity of Milk Given.

Food.	Fore-period.	Date.	Urine.			Weight.
			Nitrogen.	Creatinine.	Creatine as creatinine.	
Experiment 1. Subject M, age 14 months; underweight.						
1	*days*	*1917*	*gm.*	*mg.*	*mg.*	*kg.*
16 per cent cream, 224 cc.						
Water, 896 cc.	5	Mar. 22		70	60	6.5
Lactose, 56 gm.		" 23		72	64	
(⅕ whole milk.)		" 24		70	63	
		" 25		68	62	
7 gm. protein.			0.84	70	62	
2						
½ whole milk + lactose	5	Apr. 2		76	75	6.6
47 gm., 1,120 cc.		" 3		70	68	
		" 4		68	61	
		" 5		69	64	
18 gm. protein			2.14	71	67	
3						
Whole milk + lactose 20	2	Apr. 18		72	97	6.7
gm., 1,120 cc.		" 19		71	93	
		" 20		76	89	
36 gm. protein.			4.56	73	91	
4						
Whole milk + lactose,	14	Apr. 30		80	110	6.7
continued.		May 1		79	107	
		" 2		81	99	
		" 3		82	120	
		" 4		74	117	
			4.62	79	111	
5						
Whole milk + lactose,	22	May 8		81	125	6.8
continued.		" 9		81	124	
			5.08	81	124	

TABLE I—*Concluded.*

Effect on Creatine Excretion of Decreasing the Quantity of Milk Given.

Food.	Fore-period.	Date.	Urine.			Weight.
			Nitrogen.	Creatinine.	Creatine as creatinine.	
Experiment 2. Subject G, age 10 months; normal infant.						
1	*days*	*1917*	*gm.*	*mg.*	*mg.*	*kg.*
Whole milk + lactose 20 gm., 900 cc.	13	May 11		84	109	6.8
		" 12		82	108	
30 gm. protein.			4.37	83	108	
2						
16 per cent cream, 390 cc.	2	May 15		72	79	6.5
Water, 730 cc.		" 16		71	69	
(¼ whole milk.)		" 17		69	53	
		" 18		61	49	
12 gm. protein.			1.86	68	63	
3						
Same + lactose, 50 gm.	8	May 21		65	51	6.9
		" 22		60	40	
		" 23		63	49	
			1.20	63	47	

intermediate value. It should be noted, however, that in the case of Subject M there is still a considerable excretion of creatine when the food given is cream diluted four times. We are inclined to doubt the possibility that creatine could have been made to disappear by giving still less protein.

The fact that the increase in creatine excretion takes place quite gradually, on changing from a low to a high protein intake, appears in the tables. In the case of Subject M, the increase in creatine excretion on a high protein intake is followed over a period of 3 weeks, and even then we cannot be certain that it has reached a stationary value. It will also be noted that on changing from a high to a low protein intake (Subject G) the decrease in creatine excretion, while it takes place more rapidly, nevertheless requires

a period of days to reach a fixed level. In order to indicate the relation of this time factor to our results, we have placed in the tables, in the column marked "foreperiod," the number of days the infants were on a given protein intake before the observation period was begun.

Besides the increase in creatine excretion as the level of protein intake is raised, there is also a gradual but definite increase in creatinine elimination (Subject M). Greatly lowering the protein intake produces a well marked decrease in creatinine excretion (Subject G). A relation of protein intake to creatinine excretion is not discernible in the tables of the results of Denis and Kramer's[3] experiments.

Effect on Creatine Excretion of Varying the Amount of Whey Given.

Different levels of protein intake were obtained in the experiments just described by giving whole milk, whole milk diluted, or 16 per cent cream diluted. The infants, therefore, received various amounts of cow's milk. The resultant effect on creatine excretion is evident. However, from the results of experiments described below, it appears clearly that creatine excretion in infants, fed on cow's milk, cannot be related directly to protein intake *per se*.

Although milk has been regularly and largely used as a constituent of creatine-free diets, it has long been known that milk is not creatine-free. The quantity of creatine in cow's milk has been considered so small as to bear no appreciable relation to creatine excretion. In the case of infants, however, whose sole food is milk, and who take a relatively large volume of it, it would seem that its creatine content ought to be considered.

Using the method of Wilson and Plass,[4] we have obtained figures for preformed creatinine of ± 1.0 mg., and for creatine of 6 to 11 mg. per 100 cc. of cow's milk. Folin's method[5] may give results equally high. The older infants, with whom we experimented, received during the period of high protein feeding more than a liter of milk. If the value of creatine content given above is approximately correct, variation in the quantity of preformed

[4] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.

[5] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

creatine in the food would be sufficient to account for the difference between the quantities of creatine in the urine, when large and small amounts of cow's milk were given. We very much doubt their correctness.

Denis and Minot[6] have recently reported that the values obtained by Folin's method are twice too high. We submit, however, that it is extremely difficult to determine the quantity of creatine present in milk. We have, by various devices, tried to obtain a dependable color of sufficient intensity to read accurately, but have been quite unable to attain the conviction that we could measure the quantity of creatine present with even approximate accuracy. We have had no better success with the recently published method of Denis and Minot.[6]

In an experiment described in the preceding paper, it was found that creatine fed to an infant was excreted apparently quantitatively. This result greatly increased our suspicion that the preformed creatine present in cow's milk may be a large factor in the creatine excretion of infants. Having decided that we were unable to test this point directly by measuring the creatine content of milk, we undertook to obtain indirect evidence by observing the effect on creatine excretion of varying the amount of whey in the food. Creatine may reasonably be supposed to be in solution in milk. If this is true, its quantity in the infant's food will be proportional to the amount of whey present.

Results of Experiments.

The results of these experiments are given in Table II. In Experiments 1 and 2, three experimental diets were used. The infant was first given diluted cream, then undiluted whole milk, and, finally, diluted cream plus purified casein to such extent as to raise the protein value of the mixture to nearly that of undiluted milk. In Experiment 1 the excretion of creatine falls to the same low value on the diluted cream plus casein diet (Period 3) as when diluted cream alone is given (Period 1), in spite of the fact that the protein intake on the former diet is three times that when diluted cream alone is given. When the protein intake is increased by administration of skim milk (Period 2), the creatine excretion

[6] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxvii, 353.

TABLE II.

Effect on Creatine Excretion of Varying the Quantity of Whey in the Food.

Food.	Fore-period.	Date.	Urine.			Weight.
			Nitrogen.	Creatinine.	Creatine as creatinine.	
Experiment 1. Subject G, age 10 months; normal infant.						
1	*days*	*1917*	*gm.*	*mg.*	*mg.*	*kg.*
16 per cent cream, 390 cc.	8	May 21		65	51	
Water, 730 cc.		" 22		60	40	
Lactose, 50 gm.		" 23		63	49	
12 gm. protein.			1.20	63	47	6.9
2						
Skim milk, 1,120 cc.	6	June 19		83	135	
		" 20		84	132	
40 gm. protein.			5.30	84	133	8.2
3						
16 per cent cream, 280 cc.	5	July 6		79	44	
Water, 840 cc.						
Casein, 30 gm.		" 7		83	48	
Lactose, 60 gm.						
38 gm. protein.			3.84	81	46	8.2
Experiment 2. Subject S, age 8 months; undernourished.						
1						
16 per cent cream, 168 cc.	3	June 5		56	12	
Barley water, 672 cc.						
Lactose, 60 gm.		" 6		56	12	
(¼ whole milk.)						
5 gm. protein.			0.50	56	12	5.4
2						
Whole milk, 900 cc.	10	June 18		56	59	
		" 19		55	58	
30 gm. protein.			3.78	56	58	5.5
3						
16 per cent cream, 230 cc.	8	July 5		55	48	
Barley water, 760 cc.						
Casein, 20 gm.		" 6		53	47	
Lactose, 45 gm.						
27 gm. protein.			3.03	54	47	5.9

TABLE II—*Concluded.*

Food.	Fore-period.	Date.	Urine.			Weight.
			Nitrogen.	Creatinine.	Creatine as creatinine.	
Experiment 3. Subject X, age 16 months; underweight.						
1	*days*	*1919*	*gm.*	*mg.*	*mg.*	*kg.*
Whole milk, 1,180 cc.	5	Apr. 2		83	80	
		" 3		81	78	
		" 4		81	84	
Nitrogen, 5.34 gm.			4.41	82	81	5.9
2						
Water, 1,050 cc.	7	Apr. 22		81*	18	
Curd from 1,500 cc. milk.		" 23		90	22	
Dextri-maltose, 70 gm.		" 24		91	19	
Nitrogen, 4.20 gm.			2.60	88	20	5.9
Experiment 4. Subject R, age 2 yrs.; underweight.						
1						
Whole milk, 1,120 cc.	3	Mar. 20		78	93	
		" 21		81	89	
Nitrogen, 5.61 gm.			4.40	80	91	6.8
2						
16 per cent cream, 320 cc.	2	Apr. 9		93	105	
Whey, 800 cc.						
Lactose, 24 gm.		" 10		99	104	
Nitrogen, 2.26 gm.			1.43	96	104	7.2
3						
16 per cent cream, 320 cc.	5	Apr. 25		91	34	
Water, 800 cc.						
Casein, 24 gm.		" 26		89	34	
Lactose, 24 gm.						
Nitrogen, 4.35 gm.			3.21	90	34	6.9
Experiment 5. Subject C, age 4 months; slightly underweight.						
1						
Breast milk, 620 cc.	1	Apr. 4		46	10	
		" 5		47	11	
		" 6		45	11	
Nitrogen, 1.01 gm.			0.42	46	11	4.5
2						
16 per cent cream, 134 cc.	3	Apr. 19		55	51	
Whey, 536 cc.		" 20		60	40	
Lactose, 7 gm.		" 21		69	41	
Nitrogen, 1.15 gm.			0.81	61	44	4.6

* Computed from a 12 hr. specimen.

is markedly increased. In Experiment 2, with a similar experimental procedure, the same result is not obtained; the creatine excretion remains nearly as high on the diluted cream plus casein diet (Period 3), as during the whole milk period (Period 2). In Experiment 4, however, there is also found a much lower creatine excretion on a cream plus casein mixture than occurs when whole milk is fed. Although the protein intakes are not exactly the same, the difference is slight, and, furthermore, the differences in creatine output are out of proportion to the slight variation in protein.

In another experiment (No. 3), four times as much creatine is found in the urine on a whole milk diet as when curd without whey is given. The effect of diluting cream with whey is tested in Experiment 4 and it is found that somewhat more creatine is excreted than when the food is whole milk, containing more than double the amount of protein.

We obtained with a 4 months infant a breast milk period (Experiment 5) and find the excretion of creatine low. Breast milk protein is, in contrast with cow's milk protein, largely composed of whey proteins. This infant was then given a cream and whey mixture of nearly the same nitrogen value as breast milk, and containing casein and whey in approximately the same proportions as breast milk. The creatine excretion on this food is four times as great as on breast milk. This observation suggests that cow's milk may contain a great deal more creatine than breast milk. Inference from the results of this experiment is difficult, however, from the fact that the urine during the cream and whey period contains double the amount of nitrogen found when the food was breast milk, although the nitrogen intake was the same.

Creatinine excretion is increased in Experiment 1, when the food was changed from diluted cream to skim milk, and the same effect is obtained in Experiment 4, when whey was added to the cream. In both instances the higher value for creatinine excretion is maintained when the diet was changed to a protein intake composed mostly of casein, although on this diet there is, in these experiments, a marked decrease in creatine excretion. No effect on creatinine was observed in Experiment 2.

Discussion of Results.

The most obvious inference to be obtained from these results is that creatine contained in cow's milk may be a large factor in the creatinuria of infants fed on modification of cow's milk. We are altogether uninformed as to the actual value for creatine in cow's milk, and therefore admit the possibility of the presence in the whey of a precursor of creatine. One point clearly brought out is that the creatinuria of infants cannot be directly referred to the total protein value of the milk given. We do not wish to argue that the values for creatine excretion obtained by Denis and Kramer[1] in children may have been due to the milk portion of the high protein diet used. We feel, however, that the as yet undetermined value for the creatine content of milk makes somewhat uncertain the interpretation of the significance of creatinuria when milk forms part of a supposedly creatine-free diet.

The increased creatinine excretion on whole milk and on cream and whey may very well have been due to the larger amount of creatinine in the food than was the case when diluted cream was given. The fact that the creatinine excretion remained high, when the high protein intake was composed nearly altogether of casein (Experiments 1, 2, and 4), requires some other explanation. We can only suggest with diffidence that it is conceivable that an increase in endogenous metabolism may have been caused by the effect on basal metabolism of the high protein intake. The specific dynamic effect of protein has been shown to be very large in infants.[7]

Steenbock and Gross[8] have reported an increase of creatinuria in starving pigs when they are given casein. The results of our experiments on infants are not in agreement with this finding. Such a discrepancy of result is not, however, surprising when the wide difference in experimental conditions is taken into consideration. The basis of Steenbock and Gross' experiments is a starving metabolism, whereas the subjects of our experiments received an adequate caloric intake.

[7] Howland, J., *Tr. 15th Internat. Congr. Hyg. and Demog.*, 1913, ii, pt. 2, 438. Hoobler, B. R., *Am. J. Dis. Child.*, 1915, x, 153.

[8] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxvi, 265.

SUMMARY.

We present the results of experiments which indicate that the creatine excretion of infants bears a relation to the quantity of cow's milk fed, and also results of other experiments which indicate that the quantity of whey given is more directly related to the degree of creatinuria than is the total protein value of the food.

On the assumption that preformed creatine in milk modifications is in proportion to the quantity of whey present, our results suggest that the ingestion of creatine is probably a large factor in the creatinuria of infants fed on cow's milk. The desirability of a more definite knowledge of the creatine content of cow's milk is indicated.

Note.—Since this paper was completed for publication Denis and coworkers have published creatine and creatinine determinations on human and cow's milk (*J. Biol. Chem.*, 1919, xxxviii, 453; xxxix, 47), using the method of Denis and Minot.[6] The amounts found are smaller than those obtained by earlier methods, yet, on the basis of the above work, are sufficient to produce changes in urinary creatine upon varying the quantities of whole milk fed to infants. The suggestion above, from an isolated experiment, that cow's milk may contain more creatine than human milk, is not borne out by Denis' figures.

PLACENTAL FEEDING AND PURINE METABOLISM.

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In a previous paper Harding and Fort[1] made the suggestion that if the placenta played an active part in the nutrition of the fetus such a rôle might perhaps be found in its influence on the purine metabolism. This was suggested by the fact that their placental preparation proved to be unusually rich in arginine when compared with other organs of the human body, and also by the fact that Ackroyd and Hopkins[2] had adduced evidence to show that arginine and histidine could be regarded as the precursors of synthetic purines in the growing rat.

EXPERIMENTAL.

In order to obtain experimental evidence either for or against the supposition, it was decided to institute a series of feeding experiments in which the influence of the placenta could be contrasted with that of other proteins which might possibly serve as reserve sources of one or more amino-acids for the fetus. Placental feeding was compared with muscle feeding, and the effect on the excretion of allantoin and uric acid noted in the growing dog (not in the adult). The puppy was chosen as the experimental animal, as it gave us the opportunity to use the meat powder and placenta in moderate quantities; moreover, the quantity of food could be readily controlled, and a sufficient amount of urine was available for daily analysis. In addition, we wished to obtain evidence on the condition of creatinuria which is present in puppies and kittens according to Closson,[3] and our experiments were thus

[1] Harding, V. J., and Fort, C. A., *J. Biol. Chem.*, 1918, xxxv, 29.
[2] Ackroyd, H., and Hopkins, F. G., *Biochem. J.*, 1916, x, 551.
[3] Closson, O. E., *Am. J. Physiol.*, 1906, xvi, 252.

designed for a double purpose. Our experiments on creatinuria will be reported in a later communication. The kitten was found to be unsuited as an experimental animal. Confinement in a metabolism cage soon causes loss of appetite in the most robust animal. Even with the puppy it was found advisable to institute a "free" day every 3 or 4 days to insure a continuance of health. On the "free" day the puppy received the experimental diet but was not confined in the metabolism cage. The puppies used were usually between 3 and 4 months of age. Before the experiment they were kept on an initial purine-free diet, mainly of bread and milk. Their weight was recorded at regular intervals and showed that they were making satisfactory growth before and during the experimental periods.

A preliminary investigation, in which a number of puppies was allowed to eat a diet of bread, milk, and potatoes *ad libitum*, and on which they made satisfactory growth, showed us that about 100 calories per kilo of body weight could be considered as ample. Our experimental diets were consequently based on this figure. The following diets were used.

Diet.	Weight.	Content.			Calories.
		Protein.	Carbohydrate.	Fat.	
	gm.	gm.	gm.	gm.	
Initial purine-free		16.5	62.6	8.6	404.3
Milk	200				
Bread	100				
Meat		15.6	75.0	0.3	373.8
Potato	250				
Dextrin*	35				
Meat powder	15				
Placenta		15.6	75.0	0.4	373.9
Potato	250				
Dextrin*	35				
Placenta powder	15				

* Dextrin was sometimes replaced by corn-starch and occasionally in part by cane sugar.

The day's rations were divided into two portions, supplied in the morning and evening, and were greedily eaten by the puppies. The potatoes were always very thoroughly boiled, and whenever corn-starch was

used it was made into a paste before being mixed with the remainder of the diet. These details, although seemingly insignificant, proved to be essential to the success of the experiments.

Meat Preparation.

The meat powder was prepared from lean round steak. All visible fat and connective tissue were cut away, and the meat was passed through a mincing machine. The minced meat was then placed in boiling water for a period of 10 to 15 minutes. By means of cheese-cloth the coagulated mass was strained from the aqueous solution and much liquid fat. It was then reminced, and boiled again for a second and a third period. By this time practically all the fat and most of the extractives had been removed. After the last filtration, the remainder was spread out on coarse filter paper and rapidly air-dried at ordinary temperature. The brittle, dark brown material remaining was then ground to a fine powder in a mill and kept ready for use in tightly stoppered bottles. When wanted for use, the amount required was weighed out and allowed to absorb water before adding it to the potato and dextrin or corn-starch. A determination of nitrogen by the Kjeldahl method showed 13.6 per cent of the dried substance.

Placenta Preparation.

The placenta,[1] immediately on delivery, was placed in a jar of sterile salt solution, covered by a layer of toluene, and the whole brought to the laboratory within a few hours. The fresh placenta was first washed free from large masses of clotted blood. Then the umbilical cord and free membranes extending beyond the mass of chorionic villi were cut off as short as possible. The placenta was next placed ventral side up in a large basin and the remaining membrane dissected away. It was found possible by this means to remove practically all the large blood vessels. The remaining mass was thoroughly washed under the tap, then torn up into small pieces and again washed, and finally strained through cheese-cloth. The product was then finely minced in a mincing machine, and the remaining blood was removed by placing this material in tall glass jars through which a stream of cold water constantly circulated. To prevent loss of material a piece of cheese-cloth was tied over the top of the jar. The washing occupied from 6 to 12 hours, depending on the condition of the placenta. The washed mass was filtered through cheese-cloth and then coagulated by immersion in boiling water, faintly acidified by acetic acid, for a period of 10 minutes. At this stage the coagulated placenta was again passed through a mincing machine and subjected to further boiling in water for another two periods of 10 minutes each. After the final boiling

[1] The placentas were obtained from the Montreal Maternity Hospital, and our thanks are due to the authorities for their kindness and cooperation.

the mass was filtered at the pump, washed with distilled water, and air-dried. It was then ground to a fine powder in a mill, so that it passed through an 80 mesh sieve, and was preserved in stoppered bottles until required. About 1 kilo of material was thus prepared, representing forty-three placentas. A determination of nitrogen showed 13.7 per cent of the dry weight.

Total Purine Determination.

The two experimental diets are not purine-free. Both the meat and placenta contain nucleoproteins and water-soluble purines. The total purine content of muscle is given by Burian and Schur[5] as about 0.055 gm. of purine nitrogen per 100 gm. of fresh tissue. Placenta has been examined by Wells and Corper[6] who found 0.057 gm. of purine nitrogen in 100 gm. of fresh tissue. Thus, in the fresh state, the two tissues have practically identical purine contents. In both our diets, however, washing of the protein had been resorted to, and we felt it incumbent upon us to determine directly the purine content of our meat and placental powders.

30 gm. of meat or placental powder were boiled under a reflux condenser with 1 liter of 6 per cent sulfuric acid for 18 hours. The residue was filtered off and submitted to a further hydrolysis with 500 cc. of 5 per cent sulfuric acid for 12 hours. A slight residue from this hydrolysis was neglected. The filtrates were then neutralized separately by sodium hydrate and brought back to a condition of slight acidity with dilute acetic acid. They were next separately treated at the boiling point with copper sulfate as a 10 per cent solution and saturated sodium bisulfite solution, added alternately until a pronounced precipitate of cuprous oxide was visible. The filtrate from the second hydrolysis gave very little precipitate. The cuprous purine precipitates were united and decomposed in warm water by saturation with hydrogen sulfide. The filtrate and washings from this decomposition were next evaporated down to about 500 cc., and the purines again precipitated by copper sulfate and saturated sodium bisulfite solutions. The decomposition of the cuprous compounds was carried out as before, and the purine filtrates were concentrated to about 75 cc. This was washed into a 100 cc. volumetric flask quantitatively and diluted to the mark. A determination of nitrogen in the solution was made on aliquot parts by the Kjeldahl method and the results calculated as purine nitrogen.

30 gm. of meat powder gave 0.0360 gm. of N = 0.9 per cent of total N of meat.

30 gm. of placental powder gave 0.060 gm. of N = 1.47 per cent of total N of placenta.

[5] Burian, R., and Schur, H., *Arch. ges. Physiol.*, 1900, lxxx, 241.

[6] Wells, H. G., and Corper, H. J., *J. Biol. Chem.*, 1909, vi, 469.

The two experimental diets thus differed in their purine content by about 12 mg. per day. This small difference is insufficient to influence our results, for the differences which we have observed are far beyond that figure. In order to follow the influence of the placenta feeding upon the purine metabolism, we have determined the excretion of allantoin and of uric acid.

Urine Analysis.

The total nitrogen was determined by the Kjeldahl-Gunning process. The allantoin was determined by the process proposed by Plimmer and Skelton.[7] We have found this method, or rather our variation of it, very useful; and if the allantoin is not too small in amount, it gives an accurate determination. The Folin magnesium chloride method for the determination of urea hydrolyzes urea and allantoin into ammonia which, together with the preformed ammonia, is distilled into an excess of standard acid from an alkaline solution. The urea and preformed ammonia were determined by the urease method devised by Van Slyke and Cullen.[8] The difference gives the allantoin in terms of ammonia. We have modified the Folin magnesium chloride method[9] to suit our own convenience. In its original form, in our hands, it always required constant attention. Our modification, though a little more time-consuming, can be left unattended while other work is proceeding.

Pipette 5 cc. of urine into a 200 cc. Erlenmeyer flask, and add 5 cc. of concentrated HCl from burette and 20 gm. of crystalline magnesium chloride, together with a small cube of paraffin wax about the size of a marble. The magnesium chloride should be ammonia-free or have its ammonia content determined by blank tests. It has been our experience that the commercial salt is more likely to be ammonia-free than the so called C. P. high priced article. A reflux condenser about 45 cm. in length and 1 cm. in diameter is attached to the flask and a large safety thistle tube to the upper end of the condenser. The safety tube also carries at the top a stopper with a glass tube bent at right angles. Without these safeguards we found it impossible to avoid loss by

[7] Plimmer, R. H. A., and Skelton, R. F., *Biochem. J.*, 1914, viii, **70**, 641.
[8] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.
[9] Folin, O., *Am. J. Physiol.*, 1905, xiii, 46.

spraying. Having the condenser jacket empty, a medium sized Bunsen flame is played over the bottom of the flask until the contents boil fairly vigorously. Regulating the flame to maintain this rate of ebullition, the evaporation is continued until marked frothing occurs. This takes about 5 to 10 minutes. By gradually reducing the size of the flame, the frothing is easily controlled, and the concentration is continued until the flame of 1 inch in height is reached. This heat just serves to keep the solution boiling and is maintained for a period of 2 hours. Occasionally very little frothing occurs, and in these cases the concentration is continued until each drop of liquid falling back from the condenser causes a marked spattering of the contents of the flask. Boiling gently for 2 hours continues from this point. The concentration to be aimed at is just short of saturation of magnesium chloride at that temperature. We have found that the primary boiling hydrolyzes the urea, and the second the allantoin. During the second period of boiling under constant conditions, a stream of cold water is run through the condenser. During this second period the determination requires no attention, as there is no danger under these conditions of the liquid in the flask becoming alkaline. At the end of 2 hours, the flask is allowed to cool, and the ammonia determined as in an ordinary Kjeldahl operation, using 10 cc. of 10 per cent sodium hydrate to render the contents of the distillation flask alkaline.

In order to prove the accuracy of this procedure for the determination of allantoin, it was deemed advisable to make tests on various concentrations of pure urea and allantoin solutions. The urea was determined by the urease method of Van Slyke and Cullen, while the Kjeldahl method of total nitrogen estimation served to check the mixed solutions. 2.5013 gm. of pure urea were dissolved in 100 cc. of distilled water and 0.5005 gm. of allantoin was dissolved in a like volume of water.

Urea determinations.

1. By urease method on 0.5 cc. of solution.

HCl N/20 cc.	NaOH N/20 cc.	Urea N mg. per cc.
10	1.65	
10	1.65	
10	1.58	
10	1.58	
Average		11.69

2. By Kjeldahl method on 5 cc. of solution.

HCl N/10 cc.	NaOH N/10 cc.	Urea N mg. per cc.
50	8.73	
50	8.70	
Average		11.69
Calculated		11.68

Urea plus allantoin.

1. By modified method, using 5 cc. of urea + 5 cc. of allantoin solutions.

HCl N/10 cc.	NaOH N/10 cc.	N mg. per cc.
55	6.95	
55	6.90	
Average		13.52

2. By Kjeldahl method.

HCl N/10 cc.	NaOH N/10 cc.	N mg. per cc.
50	2.00	
50	2.10	
Average		13.54
Calculated		13.47
Allantoin determined		1.83
" calculated		1.79

The pure solutions were next diluted ten times with distilled water and the determinations repeated omitting the Kjeldahl estimations.

Urea determinations.

1. By urease method on 0.5 cc. of solution.

HCl 0.01973 N cc.	NaOH 0.02475 N cc.	N mg. per cc.
25	18.22	
25	18.24	
Average		1.157
Calculated		1.168

Urea plus allantoin.

1. By modified method on 5 cc. of urea + 5 cc. of allantoin.

HCl N/10 cc.	NaOH N/10 cc.	N mg. per cc.
10	3.80	
10	3.85	
Average		1.342
Calculated		1.347
Allantoin determined		0.185
" calculated		0.179

TABLE I.

Urine Analysis.

Puppy W.

Meat diet.							Placenta diet.						
Date.	Total N.	Urea N.	Creatinine N.	Allantoin N.	Uric acid N.		Date.	Total N.	Urea N.	Creatinine N.	Allantoin N.	Uric acid N.	
1918	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		*1918*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Apr. 6	1,099	784.3	25.38	24.98	1.45		Apr. 13	Free day.					
" 7	1,032	651.2	24.83	72.80	0.33		" 14	1,615	1,154	25.65	92.45	9.25	
" 8	1,689	1,113	24.30		1.00		" 15	1,503	955.4	25.70	215.01	8.44	
" 9	Free day.						" 16	1,908	981.8	26.55	218.5	11.24	
" 10	1,176	851.1	26.3	21.45	1.80		" 17	Free day.					
" 11	1,153	825.4	22.36	96.00	0.0		" 18	1,764	1,214	32.08	165.5	9.77	
" 12	1,274	890.4	27.87	60.24	2.70		" 19	1,433	1,030	32.15	43.05	6.42	
Average.....				55.09	1.21		.....				146.91	9.02	

TABLE II.

Urine Analysis.

Puppy G.

Meat Diet.						Placenta diet.					
Date.	Total N.	Urea N.	Creatinine N.	Allantoin N.	Uric acid N.	Date.	Total N.	Urea N.	Creatinine N.	Allantoin N.	Uric acid N.
1918	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*1918*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Apr. 6	1,033	626.1	20.27	42.55	0.23	Apr. 13	Free day.				
" 7	881	591.7	17.18	75.63	0.0	" 14	1,241	861.4	20.42	103.95	1.18
" 8	1,236	797.3	22.61	96.90	0.74	" 15	1,248	838.5	27.87	248.6	3.46
" 9	Free day.					" 16	1,319	864.4	24.05	107.2	4.18
" 10	1,172	828.7	24.38		0.0	" 17	Free day.				
" 11	1,180	960.5	24.05	136.50	1.67	" 18	1,122	893.0	25.37	71.29	5.11
" 12	1,293	916.4	24.50	52.65	1.83	" 19	1,193	837.0	22.73	62.40	3.76
Average				80.85	0.74	.....				118.70	3.54

TABLE III.

Urine Analysis.

Puppy N.

Meat diet.						Placenta diet.						Meat diet.					
Date.	Total N.	Urea N.	Creatinine N.	Allantoin N.	Uric acid N.	Date.	Total N.	Urea N.	Creatinine N.	Allantoin N.	Uric acid N.	Date.	Total N.	Urea N.	Creatinine N.	Allantoin N.	Uric acid N.
1918	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*1918*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*1918*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
June 24	1,526	1,175	21.16	94.0	4.73	June 30	Puppy taking food normally.					July 7	Free day.				
" 25	1,512	1,075	20.16	117.6	5.25												
" 26	1,606	1,161	20.66	67.50	4.97	July 1	1,637	1,056	21.39	267.1	9.48	" 8	1,263	858	24.83	123.3	5.75
						" 2	1,758	1,224	24.23	228.0	8.27	" 9	1,097	768	26.20	112.0	5.11
" 27	Free day.					" 3	1,541	988	23.10	241.0	8.46	" 10	1,343	974	29.64	98.7	5.71
" 28	1,602	1,050	20.34	141.7	5.14	" 4	Free day.										
" 29	Puppy not taking food.					" 5	1,474	1,037	27.36	182.0	8.75						
						" 6	1,496	960	28.78	182.0	8.15						
Average				105.2	5.02					220.2	8.62					111.0	5.5

Uric acid was determined colorimetrically by the Folin-Macallum[10] method as modified by Benedict and Hitchcock.[11] In the earlier part of the experiments, we were much troubled by turbidity arising in the solutions, but on using the modified Benedict uric acid reagent,[12] this difficulty disappeared.

As a check on the collection of urine, creatinine was determined by the micro method of Folin,[13] using creatinine zinc chloride as a standard, and picric acid purified according to the method of Folin and Doisy.[14] The creatine determinations also formed an essential part of the investigation on creatinuria. The urines were preserved under toluene and all analyses were carried out within 36 hours. With the diets adopted we were untroubled by any fecal contamination of the urine. Glucose was absent from all urines as judged by Benedict's qualitative test. Likewise acetoacetic acid was never observed, although tested for by the nitroprusside method of Harding and Ruttan.[15] The results are given in Tables I to III.

The protocols of the allantoin determinations and the weight records are appended in Tables IV, V, and VI.

TABLE IV.

Weight Records.

Date.	Puppy W.	Puppy G.	Date.	Puppy N.
1918	*gm.*	*gm.*	*1918*	*gm.*
Apr. 3	2,935	2,650	June 20	2,840
" 6	3,010	2,695	" 26	3,105
" 10	3,100	2,730	" 30	3,140
" 13	3,165	2,790	July 4	3,160
" 17	3,320	2,855	" 8	3,250
" 20	3,400	2,975	" 11	3,460
			" 15	3,270
			" 17	3,410

[10] Folin, O., and Macallum, A. B., *J. Biol. Chem.*, 1912, xi, 265.
[11] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.
[12] Neuwirth, I., *J. Biol. Chem.*, 1917, xxix, 478 (Note 4).
[13] Folin, O., *J. Biol. Chem.*, 1914, xvii, 469.
[14] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916-17, xxviii, 349.
[15] Harding, V. J., and Ruttan, R. F., *Biochem. J.*, 1912, vi, 445.

TABLE V.

Allantoin Determinations.

Date.	Puppy W.			Puppy G.		
	HCl N/10	NaOH N/10	Allantoin N.	HCl N/10	NaOH N/10	Allantoin N.
1918	*cc.*	*cc.*	*mg. per cc.*	*cc.*	*cc.*	*mg. per cc.*
Apr. 6	25	1.90	0.18	25	1.70	0.38
	25	1.87		25	1.68	
" 7	25	3.52	0.56	30	4.20	0.79
	25	3.50		30	4.20	
" 8				25	3.25	0.58
				25	3.20	
" 10	25	7.06	0.11			
	26	8.13				
" 11	25	13.80	0.30	25	13.80	0.42
	25	13.85		25	13.75	
" 12	25	9.50	0.25	25	9.20	0.27
	25	9.55		25	9.30	
" 14	50	4.58	0.86	25.5	2.85	0.63
	50	4.60		25.5	2.83	
" 15	75	18.75	2.47	25	3.43	1.28
	75	18.70		25	3.47	
" 16	25	28.30	0.87	25	8.50	0.46
	25	28.32		25	8.30	
" 18	25	0.37	0.77	25	6.20	0.36
	25	0.37		25	6.30	
" 19	25	3.69	0.21	25	2.85	0.40
	25	3.65		25	2.90	

TABLE VI.

Allantoin Determinations.

Date.	Puppy N.			
	HCl N/10	NaOH N/10		Allantoin N.
		1	2	
1918	*cc.*	*cc.*	*cc.*	*mg. per cc.*
June 24	25	12.15	12.20	0.201
" 25	25	3.15	3.15	0.492
" 26	25	4.30	4.20	0.250
" 28	25	3.50	3.45	0.545
July 1	25	13.45	13.35	0.490
" 2	25	10.70	10.70	0.480
" 3	25	8.70	8.65	0.700
" 5	25	11.85	11.95	0.449
" 6	25	12.25	12.25	0.462
" 8	25	15.05	15.00	0.274
" 9	25	16.45	16.65	0.228
" 10	25	11.70	11.70	0.286

DISCUSSION.

It is evident from an inspection of the figures given in Tables I to III that the feeding of placenta resulted in an increase in the excretion of both allantoin and uric acid. The smallest rise is shown by Puppy G, but even here the result is beyond the range of experimental error. Large fluctuations occur in the allantoin excretion; so large that in two cases the variations overlap in the two experimental periods, and at first led us to believe that there was something fundamentally wrong with our analytical technique. A revision of this, however, failed to show any serious error from this source except that it was realized afresh that extreme care must be taken over the allantoin determinations when there was only a small amount present. As a further check, however, the uric acid was determined. This, in the case of Puppies W and G, was made some weeks after the other analyses, but in Puppy N it was carried out concurrently with them. As the uric acid figures show a similar increase during the period of placental feeding, we were confirmed in our opinion that the latter diet resulted in an increase in purine metabolism when contrasted with meat or muscle feeding. Furthermore, in Puppy N a return to the original meat diet led to a prompt drop in the allantoin and uric acid to their original level. The increase on the placenta diet is too large to be ascribed to the difference in purine content of the two diets. In the meat diet 18 mg. of purine N were supplied per day; in the placenta diet this was increased to 30 mg. per day, giving the small difference of 12 mg. of purine N in favor of the placenta diet. This was the same for each puppy. The total increase in purine N estimated in the urine (allantoin N plus uric acid N) was 41.65, 99.63, and 118.60 mg. respectively for Puppies G, W, and N.

The possibility, too, of increased purine catabolism due to mere stimulation may also be dismissed. Both diets possessed the same nitrogen content, both were equally well absorbed, and similar amounts of nitrogen were found in the urines of the two periods. Both meat and placenta had been deprived of their extractives by water. It would appear then that the cause of the difference in the purine excretion under the two diets must be sought in their amino-acid make-up.

In an important paper entitled "Feeding experiments with deficiencies in the amino-acid supply: arginine and histidine as possible precursors of purines," Ackroyd and Hopkins,[2] as mentioned before, came to the conclusion that these two amino-acids either together or separately can act as the raw material for the synthesis of purines in the animal body. It is a generally accepted fact that such a synthesis occurs, and naturally the presence of the glyoxaline ring in both purines and histidine, and a similarity of arrangement of carbon and nitrogen atoms in arginine and the pyrimidine nucleus, had long ago pointed to these two particular amino-acids as the probable source of such a synthesis. Abderhalden together with Einbeck[16] and Schmid[17] had attempted to obtain experimental evidence in favor of such a connection without success. Ackroyd and Hopkins owe their positive evidence to a better selection of experimental conditions. They point out that in the adult animal such a synthetic process is of necessity at a minimum, and the evidence may well escape observation. Also that to supply an animal either in full nutrition or in a fasting condition with a large amount of an amino-acid suddenly is to supply it in excess of its current needs and to insure its catabolism by the most rapid path. If there now exists any alternative, a path involving a synthesis would be avoided.[18]

In their evidence, Ackroyd and Hopkins observe the effect on growth and upon the excretion of allantoin in the growing rat of the withdrawal of arginine and histidine from the diet. The original diet, which was entirely adequate, contained caseinogen as its protein. The caseinogen was hydrolyzed by acid and tryptophane and cystine was added, thus rendering it complete in regard to its amino-acid make-up. On this diet the rats made satisfactory though not entirely normal growth. The removal of arginine and histidine led to a prompt loss in body weight and a

[16] Abderhalden, E., and Einbeck, H., *Z. physiol. Chem.*, 1909, lxii, 322.

[17] Abderhalden, E., Einbeck, H., and Schmid, J., *Z. physiol. Chem.*, 1910, lxviii, 395.

[18] The acceptance of this argument involves the assumption of the rapid adaptability of the organism. Otherwise the presentation of a large amount of a particular amino-acid would involve its metabolism by all paths in accordance with the active masses of material and the relative rates of the chemical reactions.

fall in the allantoin excretion of 40 to 50 per cent. The withdrawal of only one of the two amino-acids, however, was followed by maintenance of weight, occasionally with a slight rise, and a drop in the allantoin excretion of about 17 per cent. It would seem as though arginine or histidine acted independently as an essential amino-acid, and functioned as a raw material for the synthesis of the purine ring in the growing rat. The fall in allantoin excretion is not a direct outcome of the drop in body weight occurring on the withdrawal of arginine and histidine, as the removal of trytophane from the diet occasions a loss in weight without a corresponding decrease in the allantoin excretion, although irregularities in the purine metabolism were noticed in one case.

In attempting to contrast our two experimental diets, we were surprised to find no analysis of lean round steak made according to the Van Slyke method. We therefore utilized as the basis of our calculations the results of Thrun and Trowbridge[19] on a veal composite. In Table VII is given the amino-acid content of meat and placenta diets calculated in this way. We have also included the average results of two analyses of human skeletal muscle made by Drummond.[20] The figures for the veal composite and human muscle do not differ markedly.

An inspection of the table shows that the meat diet is high in lysine as compared with the placenta diet and low in arginine. Taking its arginine and histidine content together, the placenta diet represents an increase of 50 per cent. As no special function has as yet been discovered for lysine, and as the results show that an interpretation of our experimental figures must be sought in an increment in the placental diet, we are inclined to look upon our results as strongly supporting those of Ackroyd and Hopkins. We should also like to emphasize that in our experiments the uric acid excretion was increased, as well as the allantoin excretion, during the period of placental feeding. This we think important in view of the results of Benedict[21] on the purine metabolism of the Dalmatian hound in which the allantoin and the uric

[19] Thrun, W. E., and Trowbridge, P. F., *J. Biol. Chem.*, 1917, xxxiv, 343.

[20] Drummond, J. C., *Biochem. J.*, 1916, x, 473.

[21] Benedict, S. R., *J. Lab. Clin. Med.*, 1916–17, ii, 1.

acid do not always follow parallel excretory paths. The increase in the uric acid shows unmistakably, in our minds, a synthesis of an actual *purine* ring.

Moreover, it should be again pointed out that in our experiments the animals were in full nutrition, as evidenced by their growth records. The high arginine and histidine diet resulted in an increase in the synthetic processes. In this respect our experiments differed markedly from those of Ackroyd and Hopkins, and our results are not in accordance with some of the postulates expressed in that paper.

TABLE VII.

Nitrogen partition.	Placenta (Harding and Fort).[1]	Placenta diet.	Veal composite* (Thrun and Trowbridge).	Meat diet.	Human skeletal muscle (Drummond).
	per cent	*mg. per day*	*per cent*	*mg. per day*	*per cent*
Arginine N	22.54	450.8	13.3	266.0	11.17
Histidine N	3.12	62.4	5.0	100.0	4.25
Lysine N	7.22	144.4	13.5	270.0	13.15
Cystine N	1.36	27.2	1.00	20.0	0.85
Monoamino N	51.15		60.50		58.00
Non-amino N	5.85				4.30

* Calculated from figures given in Table II, by Thrun, W. E., and Trowbridge, P. F., *J. Biol. Chem.*, 1917, xxxiv, 351.

Lastly, how far these results may be taken as indicative of a function of the placenta is a matter for further experimental inquiry. It is certain that the high arginine content of the placenta, its ready hydrolysis by all classes of proteoclastic enzymes,[22] and the formation of allantoin and uric acid in feeding experiments with the young dog would go to show that one of its possible functions may be to act as a reserve store of arginine for purine synthesis in the fetus. The results of Lewis and Doisy,[23] who by feeding men diets high in arginine and histidine were unable to obtain any increase in the uric acid output, need not be considered as antagonistic to our view. With adolescence may come a cessation or an impairment of the synthetic processes.

[22] Harding, V. J., and Young, E. G., *J. Biol. Chem.*, 1918, xxxvi, 575.
[23] Lewis, H. B., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 1.